Trials for Development of a Vaccine Against Caseous Lymphadenitis in Sheep

By

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A Thesis Submitted to the University of Khartoum in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Ph.D.) in Veterinary Science (Microbiology)

Department of Microbiology
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June 2010
DEDICATION

To my wife Amna

to the soul of my late father the first who
guided me..

and to all those who, helped me by
education, support and encouragement...
Acknowledgement

Thanks to Almighty Allah for giving me the health and strength to do this work.

My thanks, appreciation and gratitude to my supervisor Prof. Mohamed Taha Abdallah Shigidi for his fruitful guidance, valuable assistance, encouragement and patience during carrying out and writing this work. Thanks are also due to Prof. M. T. Musa Director, General of Animal Resources Research Corporation and Prof. A. M. Atta El Mannan of Regional Veterinary Laboratories and Animal Production Research Stations Directorate for their help and allowance to do part of this work at Nyala Veterinary Research Laboratory.

I would like to thank all colleagues at Nyala Veterinary Research Laboratory, especially Drs.: B. A. Ibraheim, F. M. Elhaj and O. A. Elgazouli. My deep gratitude is due to the technicians: Mr. S. A. Noga and Mr. A. A. Sulieman for the valuable help they offered, and to the assistants: Y. Abakar and I. M. Salih.

My thanks are also extended to all technicians of the Nyala slaughterhouse especially Mr. O. Ibrahiem for helping in samples collection.
I would like to express my thanks to the head and staff of Department of Bacterial Vaccines Production, Central Veterinary Research Laboratories, Soba for allowing to carry out some preparation of isolates in the Department. Thanks are also due to Mr. E. Abbas of the Department of Biochemistry, Faculty of Veterinary Medicine and Mr. A. Adam at the Department of Biochemistry, Central Veterinary Research Laboratories, Soba for their assistance.

My thanks are extended to Prof. A. M. El Hussein Director of Central Laboratory and Dr. Hanan Moawya head of Molecular Biology Department for permission to perform the work of PCR. I would also like to thank all the staff especially Dr. Hoyam Awad, Ms. Yossra A/Motaleb and Mr. M. A/Rahman for their keen assistance. Thanks are also due to the head and staff of Endemic Disease Institute for allowing me to make some readings in their lab. I would like to thank Dr. E. Ibraheim for his help and my colleague Dr. E. B. Ali and for the valuable assistance in the statistical analysis.

My thanks are due to all people, not mentioned here, who made this work easier with their sincere help.
Abstract

A survey of 9884 sheep (6560 live at animal markets and 3324 slaughtered in Nyala slaughterhouse ) and 4634 goats (4450 live at animal markets and 184 slaughtered in Nyala slaughterhouse) was carried out in South Darfur State, west Sudan, to determine the prevalence of caseous lymphadenitis which was 0.6% in live and 1.7% in slaughtered sheep, whereas it was 0.6% in live and 2.7% in slaughtered goats.

The distribution of 141 lesions in lymph nodes of slaughtered and live sheep was 50.4 % in prescapular, 14.9 % in parotid, 11.3% in precrural, 5.7% in mandibular, 3.5% in supramammary, 0.7% in mediastinal, and 0.7% in mesenteric lymph nodes. Lesions in other parts of the body were 11.3% subcutaneous abscesses, 0.7% abscess in a liver, and 0.7% abscess in lung. The distribution of 44 lesions in lymph nodes of live and slaughtered goats was 43.2 % in prescapular, 27.3% in parotid, 11.4% in mandibular and 9.1% in precrural lymph nodes. In other parts 9.1% subcutaneous abscesses were noted in goats.

Mass culture of a local strain of Corynebacterium pseudotuberculosis CP41 N was obtained using IBT bioreactor at pH 8, temperature 31 °C and dilution rate of 0.02 h⁻¹. The harvest was used to prepare five types of
vaccines; The first a cell wall vaccine containing 6mg/ 1 ml of protein, the second a whole cell vaccine containing 16mg/ 1ml of bacterial dry weight, the third a toxoid vaccine containing 8 mg/ 1 ml of protein the fourth a cell wall + toxoid vaccine (6mg/ 1ml+ 8mg/1ml) and the fifth a whole cell + toxoid vaccine (16mg/1ml + 8 mg/1ml). Saponin and aluminum hydroxide adjuvants were added in a concentration of 1mg/1ml to each of the different vaccines. A dose of 0.5 ml from the three first vaccines and of 1ml from the fourth and fifth ones were injected subcutaneously to each one of 5 groups of sheep of 7 animals, and a sixth group was left as unvaccinated control.

All animals were bled at weekly intervals. After one month a booster doses of the vaccines were given to the animals which were bled weekly for serological monitoring by the agglutination test. All animals were challenged subcutaneously with 5.1X10^8 CFU Corynebacterium pseudotuberculosis CP41 N one month after the second dose of vaccine. The animals were then slaughtered one month later to determine the number of abscesses in the carcasses. The results showed that the vaccine which consisted of whole cells + toxoid was the best of the five vaccines with a significant reduction (p< 0.05) in mean abscesses numbers in vaccinated animals after challenge with C.pseudotuberculosis. The percentage of protection of the group vaccinated with the whole cell+ toxoid was 75%
compared with 32.3%, 37.5%, 37.5% and 45.3% for the groups vaccinated with cell wall, whole cell, toxoid and the cell wall + toxoid vaccines, respectively.

Sixty-six isolates of *Corynebacterium pseudotuberculosis* from the sheep and goats were tested for their sensitivity to 16 antimicrobial agents. The isolates were found highly sensitive to nitrofurantoin, chloramphenicol, rifampacin, cotrimoxazole, erythromycin, and ampicillin. Moderately sensitive to methicillin, kanamycin, gentamycin and tetracycline, but were resistant to nalidixic acid, colistin, novobiocin, penicillin, cloxacillin and streptomycin.

PCR technique was used to examine 28 of the local isolates of *Corynebacterium pseudotuberculosis* using the primer targeting the 16S rRNA gene. The results showed homogeneity of the isolates with the molecular size of this gene.

The study showed that caseous lymphadenitis is prevalent in South Darfur State, which is an important area for sheep production in the country.

A vaccine consisting of whole cell+toxoid appears to be promising for protection against the disease, examination of this vaccine under field conditions is recommended.
ملخص الاتروحة

في مسح لتحديد مدى انتشار مرض التهاب الغدد الليمفاوية الجنبى في الضأن و الماعز.
في ولاية جنوب دارفور بغرب السودان تم فحص عدد 8848 رأس من الضأن (656 حي
بأسواق الماشية بنيالا و 3324 مذبوح بسلخانة نياالا) و عدد 4634 رأسا من الماعز (4450 حي
من أسواق الماشية بنيالا و 184 مذبوح بسلخانة نياالا). وجد نسبة انتشار المرض في الضأن
(0.6%) في الحي و (1.7%) في المذبوح، بينما كانت النسبة في الماعز (27%) (0.6%) في
الحي و (5%) (2.7%) في المذبوح. توزعت آفات المرض التي وجدت في الضأن الحي و المذبوح
141 افة (14.9%) في الغدد الليمفاوية قبل الكشفية 16% (50.4 %)، قبل الفخذية 16% (11.3 %)،
النكافة (14.9%) فوق الضرعية (5.3%)، الفكية السفلية (8.4%)، المنصفية (1%) و
المساريقة (1%) و في الكبد (1%) و الرئة (1.7%) و خراج ما تحت الجلد
13 (11.3%)، أما في الماعز الحي و المذبوح فقد وجدت (40 آفة) موزعة على الغدة قبل
الكشفية 19% (47.5 %)، الفكية السفلية 12% (30)، الفخذية 4% (10)، الفكية السفلية 5 % (12.5)
و خراج ما تحت الجلد 4% (10).

تم استخدام جهاز المفاعل الحيوي ولأول مرة في السودان لنتائج خلايا مركزية من
بكتريا وندية السل الكاذب من السلالة المحلية ومن هذا المنتج تم تجهيز خمسة أنواع من اللقاح
المرض وهي: لقاح حلاوة جدار الخلية (0.5 مل / 3 ميج)، لقاح الخلية الكاملة (0.5 مل / 8 ميج)،
لقاح الذوفان (0.5 مل / 4 ميج)، لقاح حلاوة جدار الخلية مضافا إليه الذوفان (0.5 مل / 3 ميج
+ 0.5 مل / 4 ميج) و لقاح الخلية الكاملة مضافا إليه الذوفان (0.5 مل / 8 ميج + 0.5 مل / 4 ميج).

تم حقن جرسين من هذه اللقاحات بفرق شير في كل مجموعة من خمس مجموعات من الضأن
تحتوي كل واحدة منها على سبع حيوانات وتركز مجموعة سادسة من غير حقن للضبط.

وتم جمع عينات دم لعينات الأماض من كل حيوان أسبوعيا لمعرفة الاستجابة المناعية بقياس
مستوى الأجسام المضادة باستخدام اختبار الراص البكتيري. بعد شهر واحد من الجرعة الثانية تم
حقن كل الضأن تحت الجلد بجرعة تحدى موحدة من نفس السلالة، بعد شهر واحد من التحدي تم
ذبح كل هذه الحيوانات و تم فحصها بحثا عن آفات المرض وتحليل النتائج إحصائياً. تم تقييم
درجة الحماية التي أحدثتها هذه اللقاحات في الحيوانات بعد التحدي. وقد وجد أن أفضل لقاح هو الخلية الكاملة مضافاً إليها الدفان، وكان الفرق معنويًا (P < 0.05) في تقليل متوسط عدد الآفات مقارنة ببقية اللقاحات، وكانت نسبة الحماية من انتشار الآفات في الحيوانات التي حققت بهذا اللقاح 75% مقارنة بـ 32.3%, 37.5% و 45.3% في الحيوانات التي تم حقنها بلقاح حلالية، لقاح الخلية الكاملة، لقاح الدفان، ولقاح حلالية الخليط زائداً الدفان على الترتيب.

تم اختبار حساسية لعدد 66 من معزولات ليكترية وتدية السل الكاذب لعدد 16 من المضادات الحيوية وقد وكانت السلالات حساسة جدا للنتروفيورانتون، كلورامفنكول، ريغاميسين، كورتيموكازازول، أريثروماسينين، والأمبيسنين، وكانت حساسة بدرجة متوسطة لكل من فيسيليمين، كاناماسين، جنتاماسين، والترايكلين، ومقاومة لكل من ناليدكسيك اسيد، كوليستين، نوفاميسين، بنسيلين، كولكساميسليف، والستروتاماسين.

استخدمت تقنية تفاعل البلمرة التسلسي في اثاث نوعية 28 سلالة ليكترية وتدية السل الكاذب وقد أظهرت نتيجة الرحلان الهلامي للحمض النووي الريبي منزوع الأكسجين لكل هذه العزلات نفس معدل المورث المستهدف لهذه البكتريا. من هذه الدراسة، نخلص إلى أن مرض التهاب الغدد الليمفاوية الجيني منتشر وسط الضرأ والمعار يظهر منطقية جنوب دار فور والتي تعتبر من مناطق الانتشار المهمة للضأن في البلاد، ونوصي بتجربة هذا اللقاح للتحصين ضد المرض على مستوى الحقل وتقييمه تحت الظروف الطبيعية.
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Results of PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis........
Introduction

Sudan is one of the largest African countries, with high agricultural potentialities and large livestock population. Under the present food crisis in the world, it is expected that the country will participate in filling the food shortage gap. Animal diseases are the main constrain facing livestock development. One of these diseases is caseous lymphadenitis (CLA) that caused by Corynebacterium pseudotuberculosis (C. pseudotuberculosis). The disease mainly affects sheep and goats and is world wide (Batey, 1986; Campell, 1982; Hein and Cargill, 1981; Lund et al., 1982). In Sudan the disease causes great economic losses due to condemnation of affected carcasses and because it affects live sheep and goats trade with the neighboring gulf states.

The disease is characterized by abscess formation in lymph nodes mainly and the internal organs. Once the disease is endemic, it is very difficult to eradicate (Brown and Olander, 1987). Antibiotics therapy is generally not effective as antimicrobial drugs are unable to penetrate the caseuated lymph nodes and abscesses. Control programmes usually entail culling of animals with recurring abscesses, isolation of affected ones, and lancing of abscesses.
Identification of infected animals is often difficult as internal abscesses, especially those of lungs, escape detection (Ellis et al., 1987) while external abscesses are difficult to detect in sheep carrying a full fleece. Vaccination against the disease seems to be the most practical measure to help in preventing the disease (Brown et al., 1986).

The objectives of this study were to:

1- Determine the prevalence of CLA in sheep and goats in South Darfur State as it one the main areas of production.

2- Apply PCR technique for identification of *C.pseudotuberculosis* isolates obtained during the study

3- Utilize IBT bioreactor for obtaining mass culture of *C. pseudotuberculosis* for vaccine production.

4- Prepare different vaccines to be evaluated for protection against CLA.

5- Determine the susceptibility of *C. pseudotuberculosis* strains isolated during the course of the study to various antibiotics.
Chapter One

Literature Review

1.1. Caseous lymphadenitis in sheep and goats:

Caseous Lymphadenitis is a disease of sheep and goats. Though *C. pseudotuberculosis* was originally identified as the causative microorganism of CLA in sheep and goats, this bacterium has also been isolated from other species, including horses, cattle, camels, swine, buffaloes, and humans (Yeruham *et al.*, 1996; Peel *et al.*, 1997; Selim, 2001; Williamson, 2001). The disease is characterized by suppuration of large lymph nodes, usually the superficial ones, but the internal nodes may also be involved in severe visceral forms of the disease (Woodruff and Gregory, 1929; Maddy, 1953; March, 1965; Knight, 1969; Nairn and Robertson, 1974; Muckle and Gyles, 1982, 1983; Muckle *et al.*, 1992; Pepin *et al.*, 1999; O’Doherty *et al.*, 2000; Williamson, 2001)

1.1.1. Clinical picture of caseous lymphadenitis:

It was stated that, signs of infection with the CLA appear slowly and sheep might be infected for years before clinical signs are evident (Lovell, 1969). The internal form of the disease is the most severe form. Abscessation may involve internal organs, commonly lungs, liver and Kidneys and their adjacent lymph nodes. Internal CLA may cause
emaciation and thin ewe syndrome and is often responsible for carcass condemnation after slaughter and inspection (Renshaw et al., 1979). External and internal forms may develop together. Different authors reported various observations on CLA infections thus, depending on the frequency of occurrence of infected lymph nodes, Woodruff and Oxer (1929) mentioned that the prescapular and the prefemoral lymph nodes were the most common sites. The lungs and their associated lymph nodes, the bronchial and mediastinal, were next. They found that lymph nodes of the upper part of the alimentary tract, and the parotid were not commonly affected. The mesenteric lymph nodes were even less commonly affected. It was stated that lymph nodes most commonly affected were bronchial, mediastinal, precrural, hepatic, supramammary, scrotal and popliteal (Maddy, 1953). Yet it was found that the most affected lymph nodes in order of frequency were the prescapular, prefemoral, superficial inguinal, ischiatric and popliteal. However, it was reported that the affected lymph nodes in order of frequency of infection were prescapular, prefemoral, popliteal, mediastinal and in very rare cases the renal (Gameel, 1974). It was observed that the lesions were mainly found in the prescapular, precrural, mediastinal, bronchial and subiliac lymph nodes (Ayers, 1977). Further more, it was reported that the prescapular, prefemoral, internal
iliac, mediastinal and bronchial lymph node and lungs were commonly affected (Williamson and Narin, 1980). On the other hand it was found that the lesions were mainly in the prescapular and precrural lymph nodes and in the adjacent subcutaneous tissues, in addition to the lungs parenchyma (Kuria and Nagatia, 1990). In the same time it was reported that the precrural, prescapular and popliteal lymph nodes were frequently affected, whereas the visceral lesions were commonly found in the lungs (Gilmour, 1990). Then again it was reported that lesions in the infected animals were mostly located in the prescapular, precrural, supramammary, inguinal and popliteal lymph nodes respectively. In severe caseous inflammatory processes, besides lymph nodes, lesions were found in lungs, heart, liver, spleen and peritoneum as well (Musa, 1998). Then it was reported that the distribution of lesions in carcases at Nyala slaughterhouse in the parotid, prescapular, precuraral, mandibular, popliteal and inguinal lymph nodes besides pyogenic lesions in the lungs and scrotal fascia (Elgaddal, 1997).

1.1.2. Geographic distribution of caseous lymphadenitis:

Caseous lymphadenitis in sheep and goats is prevalent in most parts of the world (Brown and Olander, 1987), especially in the areas where intensive sheep and goats industry is practiced. In Australia data gathered from abattoirs about prevalence were as high as 58% (Williamson and Nairn, 1980). In the
western United States, surveys revealed that it is the third leading cause for condemnation of sheep at slaughter, following emaciation and pneumonia (Maddy, 1953). In the Sudan, CLA in sheep was reported for the first time by Carr (1914) in Sennar. The disease was described as painless swelling and the causative agent as slender non-motile rods: the Priesz-Nocard Bacillus. The affected sheep were otherwise normal (Carr, 1914).

1.1.3. The causative agent of the disease:

1.1.3.1 History:

*Corynebacterium pseudotuberculosis* is the causative organism of CLA. Nocard isolated the organism for the first time in 1888 (Nocard 1889). The same organism was isolated and named *Bacillus pseudo-tuberculosis ovis* (Priesz, 1894). Then it was included in the genus *Corynebacterium*, the generic name they introduced for diphtheria bacillus and diphtheroid and given the name *Corynebacterium pseudotuberculosis ovis* (Lehman and Neuman, 1896). The organism was named *Corynebacterium ovis* (Bergy, 1923). The name *Corynebacterium ovis* was criticized because of its occurrence in animals other than sheep and suggested the name *Corynebacterium pseudotuberculosis* (Carne, 1939), which was adopted in the sixth edition of Bergy's manual (1948).
1.1.3.2. Taxonomy:

The genus *Corynebacterium* is one of the largest genera in the coryneform group. It accommodates the diphtheria bacillus and some other species pathogenic to animals. *Bergey’s Manual of Systematic Bacteriology* listed only 17 *Corynebacterium* species; however, 11 new species were defined between 1987 and 1995 (Funke *et al.*, 1997) and another 32 new species were described between 1996 and 2003. From 2001 to 2003, up to 13 new species were validly published ([http://www.bacterio.cict.fr/c/corynebacterium.html](http://www.bacterio.cict.fr/c/corynebacterium.html)). At present the genus *Corynebacterium* contain more than 60 species, the vast majority of which have been isolated from human or animal samples. Chemotaxonomically, this genus includes species that possess wall chemotype IV (arabinose, galactose, and *meso*-diaminopimelic acid (*meso*-DAP), short-chain mycolic acids (corynomycolic acids, approximately from 22 to 36 carbon atoms) and DNA G + C contents ranging from 51 to 63 mol% (Funke *et al.*, 1997). The narrower definition of the genus *Corynebacterium* has resulted in the transfer of several species (*Clavibacter, Rhodococcus*, and *Turicella*) were classified as other genera.
1.1.3.3. *Corynebacterium* and other similar genera:

The genus *Corynebacterium* was often grouped with *Mycobacterium*, *Nocardia* and *Rhodococcus* based on lipid content of their cell walls as CMNR-group (Brown and Olander, 1987). The guanine + cytosine content of the DNA of *Corynebacterium* is similar to that of the other mycolic acid-producing bacteria, *Mycobacterium* (62-70%) *Nocardia* (60 to 69%), *Rhodococcus* (59 to 69%) *Gordona* (63 to 69%) *Dietzia* (63 to 69%) and *Tsukamurella* (67 to 68%). This similarity may strengthen the consolidation of these genera into one family (Wayne and Kubica, 1986). The differences of true *Corynebacterium* from the genera *Mycobacterium* and *Nocardia* in addition to that they possessed a G + C rich DNA, they also contained N- glycol residues in glycan moiety of their cell wall (Collins and Cummins, 1986). On the other hand, differentiation of true corynebacteria from certain rhodococci could not be done by the analysis of mycolic acid alone. For instance representatives of the genus *Rhodococcus* were described as aerobic, although the true corynebacteria were undoubtfully facultative anaerobic (Goodfellow and Alderson, 1977). Furthermore, members of the genus *Rhodococcus* possessed 10- methyloctadecanoic acid (tuberculostearic), whereas true
corynebacteria lacked this fatty acid (Collins, Goodfellow and Minnikin, 1982).

1.1.3.4. Animal diseases caused by *C. pseudotuberculosis*:

*C. pseudotuberculosis* has been isolated from abscesses and suppurative lesions, in sheep and goats where it causes CLA. Woodruff and Gregory (1929) stated that it produced characteristic abscesses in lymph nodes. Usually the large superficial nodes were affected, but internal nodes may also be involved in severe visceral form of the disease.

Horses are the third species that is affected. The same organism causes ulcerative lymphangitis. This is an excoriating, suppurating inflammation of the lymphatics usually confined to the distal portion of the limb. A disease of horses recognized to be caused by *C.pseudotuberclosis* is the syndrome of chronic ventral abscesses reported in western United States (Hughes and Biberstein, 1959).

An outbreak of *C.pseudotuberclosis* infection in camels (*Camelus dromedarius*) in Saudi Arabia was reported (Radwan, El Magawry; Hawari; Albekairi; and Rebleza,1989). The authors postulated that the disease had spread from sheep and goats, which shared pastures with the camels. Also an outbreak of natural *C.pseudotuberculosi*s infection in adult camel herds in Jordan was described and reported for the first time (Hawari, 2008).
Human infection caused by *C. pseudotuberculosis* is a rare event, and most of the reported cases have been related to occupational exposure; one case involved the ingestion of raw goat meat and cow milk (Peel *et al.*, 1997). About 25 cases of infection of humans with this microorganism have been reported in the literature (Mills *et al.*, 1997; Peel *et al.*, 1997; Liu *et al.*, 2005). Peel *et al.* (1997) reviewed 22 cases, in which infected humans were generally presented with lymphadenitis, abscesses, and constitutional symptoms. Mills *et al.* (1997) described suppurative granulomatous lymphadenitis in a boy, due to contact with contaminated farm animals. Liu *et al.* (2005) reported *C. pseudotuberculosis* infection in a patient’s eye, due to an ocular implant. In most cases, the patients received antibiotic therapy and the affected lymph nodes were surgically removed (Mills *et al.*, 1997; Peel *et al.*, 1997; Liu *et al.*, 2005).

**1.1.3.5. Morphology of *C. pseudotuberculosis***:

Breed, Murry and Smith (1957) described *C. pseudotuberculosis* as slender rods, measuring 0.5-0.6 by 1-3 μm, it appears as a coccus or coco-bacillus, non-motile and non-sporulating and non-capsulating.

In a liquid culture, the organism shows less pleomorphism than in a fresh material from a natural infection and dominant forms being short coco-bacilli. In older cultures, bacillary and rarely filamentous forms have
been observed, but forms with club-headed ends and tapering blunt ends have also been described (Carne, 1939).

Breed et al. (1957) described the organism as staining irregularly Gram positive. Carne (1939) and Purchase (1944) found that the bacilli might have a number of transverse striations and granules, which were best seen by Neissler's stain. They have also found that the organism was non-acid fast, although Carne found that it was necessary to use 5% sulphuric acid to decolorize some strains grown in milk.

Hard (1969) examined *C. pseudotuberculosis* by electron microscope and found an electron dense follicular layer external to the cell wall and using ligroin and acetone methanol extractions, he had demonstrated lipid on the surface.

**1.1.3.6. Cultural characteristics:**

*C. pseudotuberculosis* is a facultative anaerobe which grows poorly on ordinary solid media and its growth is greatly improved by addition of blood or serum. The colonies on blood agar are yellowish-white, opaque flat with a matt surface after 24 to 48 hours incubation at 37°C. The colonies are surrounded by a narrow zone of beta-haemolysis. In primary cultures, it produces clear minute colonies (Daines and Austin, 1932). Because of the high lipid content, the cell wall lipids constitutes as much as
13.3% of the dry weight of the bacterial cell wall (Ioneda and Sliva, 1979), accordingly the colonies appear waxy, splatter in flame and can easily be pushed across the agar surface. In liquid media the growth is scanty with slight pellicle and this was attributed to the hydrophobic nature of the outer lipid layer (Brown and Olander, 1987).

1.1.3.7. Identification and characterization:

1.1.3.7.1. Biochemical properties:

1.1.3.7.1.1. Nitrate reduction:

Wholfeil and Weiland (1937) reported that the organism reduced nitrates to nitrites but Wilson and Miles (1964) found that nitrate was reduced by some but not all strains. It was reported that 21-79% of the strains reduced nitrates to nitrites (Cowan, 1974). Biberstein et al. (1971) stated that strains from horses were able to reduce nitrates to nitrites while those from sheep and goats did not. Muckle and Gyles (1982) examined 25 strains of caprine origin; they all failed to reduce nitrate. Barakat et al. (1984) and Brown and Olander (1987) reported that strains isolated from small ruminants tended not to reduce nitrate, whereas those isolated from horses did, and they suggested that this criterion could be used for biotyping. Restriction endonuclease assays were performed on few strains and this revealed restriction patterns that correlated with the ability or
inability of the isolates to reduce nitrate (Groman et al., 1984). The work of Groman et al. (1984) was confirmed (Songer et al., 1988) by existence of two biovars of the organism, they have suggested biovar ovis, for the nitrate-reductase negative strains and biovar equi for those that were nitrate-reductase positive.

1.1.3.7.1.2. Urease and catalase production:

It was stated that C.pseudotuberculosis was capable of hydrolyzing urea and producing catalase enzyme (Cowan, 1974). It was reported that many strains could hydrolyze urea (Hughes and Biberstein, 1959; Dennis and Bamford, 1966; Muckle and Gyles, 1982). It was mentioned that attenuated strains of C.pseudotuberculosis were urease negative (Burrell, 1979). It was accepted that C.pseudotuberculosis is catalase positive (Cowan, 1974; Songer et al., 1988).

1.1.3.7.1.3. Fermentation of carbohydrates:

C.pseudotuberculosis, like other cornynebacteria produce acid but no gas from fermentable carbohydrates. Acids are produced from glucose, maltose, galactose, fructose and mannose. Variable results were reported from sucrose, lactose, xylose, dextrin, arabinose, mannitol and glycerol (Collins and Cummins, 1986). It was mentioned that C.pseudotuberculosis strains were positive to glucose, sucrose and maltose, negative to trehalose
and salicin, variable results to lactose (Barrow and Feltham, 1993).

1.1.3.7.1.4. Other biochemical properties:

Cowan (1974) reported that *C. pseudotuberculosis* did not produce H₂S, in contrast to Dennis and Bamford (1966) who reported variable results.

Carne (1939) found slight liquefaction of gelatin. In contrast, Hughes and Biberstein (1959) examined eight strains and all were found positive. Variable results of gelatin liquefaction were reported (Barrow and Feltham, 1993).

Dennis and Bamford (1966) reported variable results for methyl red test. However, Collins and Cummins (1986) stated that the organism was positive for methyl red.

*C. pseudotuberculosis* does not produce indole (Dennis and Bamford, 1966; Cowan, 1974; Songer *et al.*, 1988).

1.1.3.7.2. API Coryne system:

A biochemical test for coryneform bacteria identification the API Coryne system (API-bioMérieux, Inc., La Balme les Grottes, France) was established (Dorella, 2006). This system consists of 21 biochemical tests which can be performed in 24–48 h. The test contains 20 tubes containing substrates that allow for 11 enzyme tests (pyrazinamidase, pyrrolidonyl arylamidase, β-galactosidase, alkaline phosphatase, α-glucosidase,
Nacetylglucosaminidase, β-glucuronidase, and nitrate reduction and gelatin, urea and esculin hydrolysis) and eight carbohydrate fermentation tests (glucose, ribose, D-xylose, mannitol, maltose, lactose, sucrose and glycogen). This system was found more reliable and rapid when it is compared with standard identification methods (API-bioMérieux, Inc.).

1.1.3.7.3. Genetic characterization:

Genetically, for characterization of *C. pseudotuberculosis* Songer *et al.* (1988) reported correlation between restriction enzyme analysis (REA) of chromosomal DNA profile and ability or inability of isolates to reduce nitrate, but numerous bands generated by this technique made interpretation of the results difficult. Sutherland *et al.* (1996) used restriction fragment length polymorphism (RFLP), they only managed to detect the differences between nitrate-positive and nitrate negative groups by this technique. Literack *et al.* (1999) tested eight isolates and two reference strains for biomedical reactions, antibiotics susceptibility, production of phospholipase D, and restriction endonuclease profile and ribotyping. Both phenotypic and genotypic properties of the tested and reference strains were identical, and all of them were identified as *C. pseudotuberculosis*. Conner *et al.* (2000) characterized 50 isolates from sheep and horses, an original goat outbreak strain and a type strain by biotyping, antimicrobial susceptibility, production of phospholipase D (PLD), and
genotypically by pulsed-field gel electrophoresis using SFIL and SMAL endonucleases. All the isolates were confirmed as C. pseudotuberculosis, and all produced PLD but none reduced nitrate. Restriction with SfiI generated 16 – 18 bands between 48.5 and 290 kb and differentiated six pulsotypes.

More recently, analysis of partial gene sequences from the β-subunit of RNA polymerase (rpoB) has been shown to be more accurate for the identification of Corynebacterium species than analyses based on 16S rDNA (Khamis et al., 2004; 2005). This method has also been successfully used to identify mycobacterial species (Kim et al., 1999). Although the rpoB gene is a powerful identification tool, many authors propose that it may be used to complement the 16S rRNA gene analysis in the phylogenetic studies of Corynebacterium and Mycobacterium species (Mollet et al., 1997; Kim et al., 1999; Khamis et al., 2004; 2005). Dorella et al. (2006) constructed a phylogenetic tree based on rpoB gene sequences of reference strains from the CMN group. Based on this phylogenetic tree, they could observe a clear relationship between C. pseudotuberculosis and C. ulcerans. Moreover, analysis using the rpoB gene allowed the identification of the group that these two species belong to, as previously observed by Khamis et al. (2004; 2005).
1.1.3.7.4. Polymerase Chain Reaction (PCR):

Çetinkaya et al. (2002) carried out a study to estimate prevalence of caseous lymphadenitis (CL) in sheep and goats slaughtered at a local abattoir in Turkey. A total of 2046 sheep and 2262 goat carcasses were examined during the study period and 118 lymph nodes with abscesses, 89 from sheep and 29 from goats, were collected. *Corynebacterium pseudotuberculosis* strains were isolated from 81.4% of the abscesses, giving an overall prevalence of 2.2%. The prevalence was 3.5 and 1.1% in sheep and goats, respectively. PCR on DNA extracted from 96 suspicious isolates, using a pair of *Corynebacterium pseudotuberculosis*-specific primers, was positive for 93. Although cross-reaction with *C. ulcerans*, a human/bovine species, was observed, the PCR assay used in this study may successfully be applied for the diagnosis of CL in goats and sheep as an alternative to conventional methods, owing to its advantages of specificity and speed. Pacheco et al. (2007) in an effort to facilitate *C. pseudotuberculosis* detection, developed a multiplex PCR (mPCR) assay targeting three genes of this bacterium: the 16S rRNA, *rpoB* and *pld* genes. This method allowed efficient identification of 40 isolates of this bacterium that had been identified previously by biochemical tests. Analysis of
taxonomically related species did not generate the *C. pseudotuberculosis* mPCR amplification profile, thereby demonstrating the assay's specificity. As little as 1 pg of *C. pseudotuberculosis* genomic DNA was detected by this mPCR assay, demonstrating the sensitivity of the method. The detection limit in clinical samples was estimated to be $10^3$ CFU/ml. *C. pseudotuberculosis* could be detected directly in pus samples from infected sheep and goats ($n=56$) with a high diagnostic sensitivity (94.6%). The developed assay significantly improves rapid *C. pseudotuberculosis* detection and could supersede bacteriological culture for microbiological and epidemiological diagnosis of CLA.

1.1.3.8. **Haemolysis:**

Variable degrees of haemolysis on blood agar plate culture were observed (Hall and Stone, 1916; Minnet, 1922; Carne, 1939; Dhanda and Singh, 1955; Fraser, 1961; Zaki, 1965; Lovell and Zaki, 1966; Lovell, 1969). Minnet (1922) reported that nine out of ten strains produced a narrow zone of haemolysis while one strain of low virulence did not. It was found that some strains produced beta haemolysis, some partial haemolysis and some no haemolysis on 10% defibrinated sheep blood agar (Carne, 1939). A weak haemolysis was observed on blood agar plates (Dhanda and Singh, 1955). Lovell (1969) stated that the organism produced a haemolysin. A synergistic haemolytic effect of mixed culture of
C. pseudotuberculosis and C. equi was observed on sheep, goats, ox and rabbit, but not on horse blood (Fraser, 1961). It was found that sterile supernatant of digest broth culture of toxigenic strains had haemolytic activity on blood agar plates (Zaki, 1965; Lovell and Zaki, 1966). Hall and Stone (1916) mentioned that haemolysis was inhibited by the presence of fermentable carbohydrates, while Carne (1939) found that glucose did not, and that 10% carbon dioxide had no influence on haemolysin production but anaerobiosis favoured it. It was stated that haemolysis was a separate activity of the bacterial cell and was not associated with the filterable toxin (Lovell, 1969).

1.1.3.9. Toxin Production:

C. pseudotuberculosis produces an exotoxin in vitro. Carne (1939) and Purchase (1944) described a condition which was characterized by haemolysis of red blood cells after incubation of sheep blood with liquid cultures of virulent C. pseudotuberculosis. Lovell and Zaki (1966) mentioned that not all of their strains produced a toxin while Rottgardt (1930) stated that all his isolates formed a toxin. It was observed that toxins produced by different strains were similar (Doty et al., 1964). However, not all strains were equally potent toxin producers (Daines and Austin, 1932) and there was loss of toxicity by some of the strains. Rottgardt (1930) and Carne (1940) described a technique for the production of high concentration of the exotoxin in vitro which was associated with pellicle
formation in liquid cultures. The toxin was stable in liquid form but inactivated by heat at 60°C for 10 minutes, 37°C for two weeks or 25°C for three months and by prolonged storage, in acidic environment of pH less than 5 or by formalinization. Observations revealed that the action of toxin was on the walls of blood vessels. Jolly (1965), after labelling plasma with Evans Blue observed areas of colouring at sites of intradermal inoculation of the toxin. It was also found that injection of the toxin into the vascular corneo-acletric junction in sheep produced a severe local reaction similar to that seen in the skin.

Soueek et al. (1971) reported that the toxin of *C.pseudotuberculosis* was a phospholipase D acting on sphingomyelin to produce N- acylsphingosyl phosphate (cermide phosphate) and choline. Zaki (1965) demonstrated that lysis of ox erythrocytes by Staphylococcus B-hemolysin could be inhibited by products of toxigenic strains of *C. pseudotuberculosis*. It was found that this inhibitory effect was paralleled with the toxicity as measured in vitro. The effect was believed to be the result of its action on sphingomyelin molecule, rendering them unsusceptible to subsequent attack by the phospholipase of the *Staphylococcus B*- haemolysin. Onon (1978) using ammonium sulphate precipitation, purified the toxin to about 400- fold to homogeneity and characterized it structurally to be a basic glycoprotein of molecular weight 14500 ± 1000 daltons with an amino acid composition of hydroxyproline, hydroxylisn and glycin, resembling that of
Burrell (1979) described the optimal conditions for the haemolytic activity of *C.pseudotuberculosis* exotoxin, and concluded that the exotoxin had a haemolytic activity at pH below 6 and RBCs adhesion activity at neutral or slightly alkaline pH. He also stated that the haemolytic substance passed through 0.22 μ filter, but was retained by Seitz (0.45 μ bore size) filtration. It was inactivated by heat and its activity was inhibited by immune serum. It was stated that haemoagglutination titre was closely related to haemolytic and dermonecrotic titres of *C.pseudotuberculosis* culture supernatant (Burrell, 1980c). He suggested that haemoagglutination was another activity of *C.pseudotuberculosis* exotoxin.

Alan *et al.* (1980) investigated, *in vitro*, the mechanism of synergistic lysis of sheep erythrocytes by *C.pseudotuberculosis* and *C.equi* (*Rhodococcus equi*). They reported that haemolysis required: Firstly the action of phospholipase D from *C.pseudotuberculosis*, secondly the action of extracellular protein of *C.equi*, the third was the presence of magnesium ions. The extracellular protein of *C.equi* was purified to homogeneity and was found to be phospholipase C capable of hydrolyzing ceramide phosphate, and all of the isolated major phospholipids of mammalian erythrocyte membrane. Maximum lysis was found to require the imposition
on the system of a fourth condition, such as chilling. Mg ions were required in the first and second step. They inferred that sphingomyelin of sheep erythrocytes was first converted to ceramide phosphate by *C. equi* phospholipase C, and the resultant, in situ, ceramide then underwent dislocation by chilling and perhaps also by virtue of an affinity between ceramide and *C. equi* phospholipase C, the dislocation of ceramide presumably disorganized the lipid bilayer sufficiently to result in cell lysis.

Sutherland, Sperijers and Andres (1989) compared the activity of exotoxin produced by four strains of *C. pseudotuberculosis* according to their ability to kill white mice, haemolytic activity, *Staphylococcus* haemolysin-inhibitory effect and activity in an enzyme-linked immunosorbent assay (ELISA). They reported that exotoxin with haemolytic titre of 1: 256 or more, killed all mice and had the most inhibitory effect, (1: 64 or more) on Staphylococcus haemolysis-inhibiting titre. They did not find significant correlation between the ELISA and relative toxicity of the exotoxin.

1.1.3.10. **Antimicrobial susceptibility tests:**

The susceptibility pattern of *C. pseudotuberculosis* to antimicrobial agents varies among isolates obtained from various sources (Conner *et al.*, 2000; Foley *et al.*, 2004). Isolates of *C. pseudotuberculosis* from human were
found susceptible to erythromycin and penicillin (Lipsky et al., 1982).

Muckle and Gyles (1982) in a study of 26 strains isolated from lesions of
caseous lymphadenitis in goats, reported that all strains were susceptible to
the antibiotics ampicillin, chloramphenicol, lincomycin, gentamicin,
tetracycline, penicillin G and sulfamethoxazole-trimethoprim. Only three
isolates were susceptible to neomycin, and all strains were resistant to
streptomycin. Garg et al. (1985) reported that strains of
*C. pseudotuberculosis* were strongly resistant to penicillin but susceptible
to neomycin. A strain highly resistant to streptomycin (500 ug/mL) was
observed in a study of 22 isolates of *C.pseudotuberculosis* from sheep and
goats’ abscesses. Costa et al. (1998) reported that the minimal inhibitory
concentrations (MICs) of 17 antimicrobial agents for *C. pseudotuberculosis*
were similar to those described by others (Muckle and Gyles, 1982;
Adamson et al., 1985; Prescott and Yielding, 1990; Judson and Songer,
1991). They stated that the slight variation of MIC of some antimicrobial
agents for isolates from small ruminants vs. horses and cattle has
questionable clinical significance but may be yet another indication of
differentiation between isolates obtained from different sources.

However, Fernández et al. (2001) found higher MIC value for several
antimicrobial agents in an analysis of corynebacteria isolated from ewe
mastitis. Olson et al. (2002) grew *C. pseudotuberculosis* as a biofilm, in an attempt to reproduce the environment of a natural infection. They observed that this bacterium was highly resistant to all the drugs that they tested under such growth conditions.

1.4. Pathogenesis:

Infection of superficial wounds with *C. pseudotuberculosis* represented the initial events in pathogenesis of CLA, followed by spread of infection to the regional lymph nodes which subsequently suppurate (Maddy, 1953; Jubb and Kennedy, 1970; Kimberling, 1988). It is apparent that two factors play essential roles in development of the disease. First, the high lipid content of the outer cell wall layer of *C. pseudotuberculosis* allows the organism to resist digestion by cellular enzymes and persist as facultative intracellular parasite. Hard (1972) examined the toxic effect of a petrol-ether lipid extracted from the surface coat of *C. pseudotuberculosis* on peritoneal macrophages in a mouse, a rabbit and a guinea pig. He investigated cytotoxicity, viability assayed by dye exclusion, glycolytic activity and ultrastructural morphology. The viability test demonstrated a lethal effect on normal and immune mouse macrophages but not on those of the rabbit or guinea pig. Measurement of glycolysis indicated a significant degree of cytotoxicity in normal mouse macrophage ingesting lipid, a
nonsignificant depression of activity in cells from immune mice, and no alteration in the activities of rabbit and guinea pig macrophages. Electron microscopy demonstrated that *C.pseudotuberculosis* surface lipid caused acute lethal injury in normal and immune mouse macrophages. Tashijian (1983) showed in an electron microscopic study using caprine mammary macrophages, that *C.pseudotuberculosis* remained viable within macrophages. Fusion of phagosomes was documented by histochemical localization of acid phosphatase. Despite this fusion, the organism survived and the caprine macrophages which underwent progressive degeneration and died within twenty hours. It is this ability of *C.pseudotuberculosis* which resists digestion by phagocytes that results in the eventual formation of abscess. The virulence of a particular strain may be related to the amount of cell wall lipid. The amount of surface lipids has been shown to be greater in more pathogenic strains (Muckle and Gyles, 1984). McKean et al. (2007) stated that the expression of PLD by intracellular *C. pseudotuberculosis* was shown to play a small but significant role in the reduction of macrophage viability following infection. They demonstrated that the regulation of *C. pseudotuberculosis* PLD is complex. This regulatory complexity may play an important role in allowing the pathogen to successfully adapt to the changing host environment during infection,
migration, establishment and disease progression.

Production of an exotoxin is the second important component of the pathogenic mechanisms of *C.pseudotuberculosis*. The toxin was initially isolated by Carne (1940), who described its physical properties and investigated some of its pathogenic properties. He noted that it caused an extensive spreading haemorrhagic necrosis when injected subcutaneously into rabbits and guinea pigs. Jolly (1965) designed a set of experiments and concluded that the exotoxin was important as a permeability factor and that the resulting leakage of plasma from small body vessels at the site of infection increased the probability of spread of the organism to the regional lymph nodes.

The relative contribution in the pathogenesis made by each of these two factors was investigated by Zaki (1976). He found that mice receiving viable *C.pseudotuberculosis* cells developed abscesses throughout the body, while mice given the same dose mixed with antitoxin, developed abscesses only at the portal of entry. He concluded that the cell wall lipid was the pyogenic factor, and that exotoxin was not involved in the formation of abscesses, but responsible for the spread of the organism.

Kimberling (1988), describing the pathogenesis of CLA, stated that after penetration of the organism through broken skin or mucous
membrane, the invading bacteria entered the afferent lymphatic then moved to the draining lymph nodes where leukocytes accumulated around and among the bacteria, then fibroblasts and capillaries were formed at the periphery of the site of infection. The toxic bacterial metabolites including the exotoxin, slowly killed the leukocytes and tissues. Therefore the typical lesion of the disease consisted of a central mass of necrotic tissue surrounded by a wall of connective tissues and capillaries were formed. Toxin and ischemia killed the cells of the inner part of the connective tissue wall and thus, added a new layer to the necrotic mass. Then new connective tissues were formed to reinforce the wall. Successive layers were added to the necrotic mass, by repeating this process, thus, the typical lesion of CLA was formed. Subsequent spread of the organism to the lungs and other organs via lymphogenous and haematogenous means might have occurred in some animals. But spread of the organism from respiratory system to other organs occasionally occurred (Maddy, 1953).

1.1.5. Differences of CLA in sheep and goats:

The disease primarily affects the large superficial lymph nodes of both sheep and goats. The internal form with visceral abscesses is more severe, but it is found to be less frequent in both sheep and goats. There are obvious differences in CLA between the two species, those are:
(i) There is variation in the distribution of lesions. In goats the parotid node is affected most commonly, followed by the prescapular and then by other nodes, often found in the head and neck area. The superficial lymph nodes in the caudal half of the body are infrequently affected (Ayers, 1977). In comparison the lesions in the head and neck area are relatively rare in sheep. The precrural node is the most likely to be affected, followed in frequency by the perscapular and then by other lymph nodes over all the body surface with equal frequency (Ayers, 1977).

(ii) The morphological appearance of the abscessed node differs in sheep from that in goats (Ayers, 1977). In sheep it was found that the abscessed node often had a characteristic lamellated appearance with concentric fibrous bands separated by inspissated caseous material giving onion ring configuration. In goats, abscessed nodes did not have that shape and the exudates are usually uniformly pasty rather than dry. This may be due to the more liquefactive nature of phagocytic enzymes of goats (Ashfaq and Cambell, 1979).

(iii) The occurrence of visceral form of CLA was found to be less common and less extensive in goats than that encountered in sheep (Renshaw et al., 1979); (Hein and Cargill, 1981). There is a greater
tendency for multiple internal nodes to be affected in sheep (Hein and Cargill, 1981; Musa, 1998).

1.1.6. Serological diagnosis of CLA:

Diagnosis of CLA by palpation of external lymph nodes does not detect carriers, and early cases or animals with visceral form of the disease. Also by this method, CLA can be confused with Morel’s disease. Hence, many serodiagnostic tests have been investigated, but none of these was adequate for widespread use in a programme of eradication by culling diseased or carrier animals. However, several tests have been developed for the diagnosis of CLA, most of these measure the humoral response to the exotoxin (Brown and Olander, 1987).

1.1.6.1. Skin test:

Carne (1939) developed a diagnostic test similar to the Schick test in which an amount of the necrotizing exotoxin was injected intradermally. The effect of the toxin was neutralized immediately and there was no reaction in animal having antibodies. While in absence of antibodies, spreading redness and swelling visible for several days occurred. Carne injected *C.pseudotuberculosis* intradermally into 100 sheep with CLA, irregular and independable reactions were noticed. Doty *et al.* (1964) attempted a skin test in which exotoxin was titrated with
the test serum and injected intradermally in rabbits. Absence of necrosis was an indication of the presence of antibodies.

1.1.6.2. Haemolysis inhibition test (HIT):

Haemolysis inhibition test (HIT) is a test for detection of antibodies to *C.pseudotuberculosis* exotoxin. The test based on neutralization of *C.pseudotuberculosis* exotoxin by antitoxin. Application of HIT for assay of antitoxin response had been described in preliminary reports on vaccination against *C.pseudotuberculosis* (Burrell, 1976, 1978b) and experimental infection (Burrell, 1976). Burrell (1980a) used the test for diagnosis, to detect antitoxin in 24 out of 30 sheep with proven caseous lymphadenitis on autopsy.

1.1.6.3. Bacterial agglutination test (BAT):

Agglutinating antibodies are produced in many infections and particularly those in which invasion of the blood stream takes place. Agglutinating antibodies could be obviously demonstrated if suitable antigen suspensions could be prepared. Shigidi (1979) applied the method for diagnosis of *C.ovis* infection in sheep in a comparative study. Lund *et al.* (1982) utilized the test for detecting CLA among goats. They reported that the test was easy and quick to perform. Brogden *et al.* (1984) used BAT for monitoring the serologic response in vaccinated animals.
1.1.6.4. Anti-beta haemolysis inhibition test (ABH):

Utilizing the exotoxin property of inhibition of red blood cell lysis by *Staphylococcal beta-lys*, Zaki (1968) developed the ABH test. Dilutions of a test serum were incubated with a standard amount of exotoxin and bovine erythrocytes and then staphylococcal haemolysin was added. In the absence of antibodies, *C.pseudotuberculosis* exotoxin occupied the receptor site on the erythrocyte membrane preventing the beta lysin by exerting its haemolytic effect. This method had sensitivity and specificity levels of 92% and 96%, respectively, as measured by Zaki (1968) in an abattoir survey of 200 goats and sheep.

1.1.6.5. The synergistic haemolysis inhibition test (SHI):

This test was developed by Knight (1978) for diagnosis of *C.pseudotuberculosis* infection in horses. It utilized the complementary actions of exotoxins for *C.pseudotuberculosis* and *C.equi* to cause haemolysis synergically. Brown *et al.* (1986) used the SHI test to detect unapparent CLA in sheep and goats. The test was applied to serum samples from 196 goats and 76 sheep including animals with and without abscesses. Fifty-one out of 52 (98%) goats and 27 out of 28 (96%) sheep with abscesses caused by *C.pseudotuberculosis* had seropositive titers and the seropositivity continued on subsequent sampling even after healing of
superficial lesions. It was suggested that the SHI might detect sub-clinically infected animals as well as animals with clinical lesions. In animals with no abscesses, 53 (28%) of 186 goats and 4 out of 41 sheep were seropositive. They concluded that either the SHI was lacking specificity or the titres were a reflection of past or current infection without any grossly visible abscesses.

1.1.6.6. Indirect Haemoagglutination test (IHT):

Shigidi (1978) described an indirect haemoagglutination (IHT) test for diagnosis of CLA in sheep. Purified \textit{C pseudotuberculosis} toxin was used to sensitize formalinized sheep erythrocytes treated with diazobenzidine. His results showed that the IHT was a sensitive method for detection of \textit{C.pseudotuberculosis} antibodies. The author evaluated the IHT and the anti-beta haemolysis inhibition tests for the serodiagnosis of CLA in sheep, results indicated that neither of the tests was 100% reliable.

1.1.6.7. Microagglutination test:

Menzies and Mukle (1989) examined two flocks composed of 326 and four sheep flocks of 34 animals for the presence of abscesses and antibody levels. All those animals previously suffered from CLA. The sensitivity of this method was poor to good for both species (52.3% for goats and 89.7%
for sheep). The specificity was fair to poor (64.9% for goats and 21.7% for sheep).

1.1.6.8. Immunodiffusion test (IDT):

Burrell (1980b) reported this method for diagnosis of CLA. In this test exotoxin and sera, from sheep and goats, were used as reactants in double immunodiffusion test. When this method was used for diagnosis, sera from 32 confirmed cases of CLA in sheep and goats were positive whereas sera from 16 sheep free from CLA were negative.

1.1.6.9. Enzyme – linked – immunosorbant assay (ELISA):

Sutherland et al. (1987) used two ELISA tests to measure antibody response to cell wall antigens (C-ELISA) and exotoxin antigen (T-ELISA) to detect CLA in naturally infected sheep. The sensitivity and specificity of C.ELISA was 76% and 73% respectively and for T.ELISA was 67% and 77% respectively. Kuria and Holstad (1989) correlated between haemolysis inhibition test (HIT) and the ELISA. They examined sera from 52 sheep using HIT and ELISA, results showed high correlation between the HIT and the antitoxin ELISA. Ellis et al. (1990) found that C.ELISA detected 96.9% (32/33) of sheep with CLA and T.ELISA detected 84.8% (28/33) in the same group of positive sheep. Ter Laak et al. (1992) used double antibody sandwich ELISA and immunoblot analysis to detect antibodies targeted the
exotoxins, they tested field sera to evaluate the assay on a large scale basis. The ELISA specificity and sensitivity was about 100%.

Drecksen et al. (2000) modified a double antibody sandwich ELISA (ELISA A) developed for the detection of *Corynebacterium pseudotuberculosis* to improve its sensitivity. To establish the sensitivity of this modified ELISA (ELISA B), sera from 183 sheep and 186 goats were tested using ELISAs A and B. Comparison was also made with two further ELISAs (C and D) developed in Australia respectively, that detect antibodies to cell wall antigens or toxin. ELISA B had the best performance of the four tests. Its specificity was 98 ± 1% for the goats and 99 ±1% for sheep. Its sensitivity was 94 ± 3% for goats and 79 ± 5% for sheep.

Kaba et al. (2001) developed an ELISA for the diagnosis of *Corynebacterium pseudotuberculosis* infections in goats. A bacterial whole cell extract was used as solid-phase antigen, and serum from a culturepositive animal served as the internal reference standard. The well-to-well and assay-to-assay variations were determined to be 12.7 and 33.0%, respectively. A cut off value was determined by parallel testing of 142 sera (112 ELISA-positive, 30 ELISA-negative) in a Western blot, and the correlation between both tests was highly significant (K= 0, 93). In addition, the reliability of the ELISA for the detection of infected herds was
proven in a double blind study testing 910 sera from 74 goat herds. Binns et al. (2007) developed an ELISA using sonicated \emph{C. pseudotuberculosis} and optimised to detect total antibody or IgG class antibody in serum. Receiver operating characteristic (ROC) curves were obtained and the area under the ROC curve was used to compare the sensitivity and specificity of the two ELISAs. Both versions of the ELISA were evaluated on a panel of 150 positive reference sera and 103 negative reference sera. Using the test at 100% specificity, the sensitivity of detection of total antibody was 71% (95% confidence interval 63–78%), and the sensitivity of detection of IgG antibody to \emph{C. pseudotuberculosis} was 83% (76–89%), which compares favourably with other reported ELISA tests for CLA in sheep. The sensitivity of the IgG antibody assay may be higher because of the greater affinity of IgG class antibodies compared with the IgM antibodies also detected by the total antibody ELISA. The results of ROC analysis indicated that the IgG isotype ELISA was more accurate than the total antibody ELISA. The efficiency of the test was greatest when serum samples were run in a dilution series than when any single serum dilution was used.

1.1.6.10. Blood interferon-gamma assay:
Sunil et al. (2008) validated a whole blood interferon-gamma (IFN-g) enzyme immunoassay (EIA) (Bovigam, Pfizer) in experimentally infected
sheep and in a flock of known-negative sheep, as well as in a naturally infected flock, a proportion of which was vaccinated with a commercial CLA vaccine. An optical density (OD) at (540 nm) and a cut-off of 0.09 was effective in classifying animals as test positive or negative in the naturally infected flock, although there was variation in OD between visits, notably with weakly reacting animals. The test had a sensitivity of 91% and a specificity of 98%. Postmortem data supported the results in test-negative animals. Visit-to-visit variation in IFN-g EIA OD in the naturally infected flock as well as CLA disease status was used to develop an algorithm for the eradication of CLA from a known infected flock. The whole blood IFN-g assay shows promise for eradication of caseous lymphadenitis from sheep flocks.

1.1.7. Immunization against caseous lymphadenitis:

A protective immunity for sheep to control caseous lymphadenitis was first described by Quevedso et al. (1957). Trials in Patagonia and Argentina using formalized culture of \textit{C.pseudotuberculosis} with aluminum hydroxide adjuvant to vaccinate sheep achieved a reduction in infection to up 60%. Jolly (1965) injected varying numbers of \textit{C.pseudotuberculosis} into mice. High concentrations caused an acute illness and death within three to four days due to systemic effect of the exotoxin. Small numbers of the organism caused a subacute illness, and the animals developed abscesses in internal organs. He noticed that recovery from infection was greatly associated with the presence of mature and specialised macrophages in the lesions. He
assumed a cellular type of immunity associated with the onset of acquired resistance.

Cameron et al. (1969) immunized mice with formalinized corynebacterial suspension, cell wall and protoplasm and evaluated their immunogenicity by intravenous challenge. The cell wall preparation was a good immunizing agent. He concluded that the protective antigen was a cell wall protein.

Later workers have stressed the importance of cell-mediated rather than humoral immunity in preventing the formation of abscesses. Hard (1970) demonstrated the role of cell-mediated immunity in an adoptive transfer experiment. Cameron et al. (1972) inoculated merino sheep with formalin-inactivated suspension of *C. pseudotuberculosis* culture. Vaccination protected against acute or subacute death, but did not prevent development of abscesses. Irwin and Knight (1975) also provided evidence of importance of cell-mediated immunity. Mice given levamisole and *C. pseudotuberculosis* bacterin were significantly able to resist challenge with virulent *C. pseudotuberculosis* compared to those which received bacterin alone. Levamisole enhances the differentiation of T lymphocytes and their response to antigens. The enhanced resistance was found to be associated with quantitative reduction in serum immunoglobulin levels.
Zaki (1976) demonstrated the role of the two arms of immune response and concluded that antitoxin did not interfere with multiplication of the organism but hindered its systemic spread, whereas the cell-mediated immunity restricted bacterial proliferation.

Nairn et al. (1977) vaccinated 24 weaner sheep with formalinized concentrated crude exotoxin, then challenged vaccinated and control animals. All vaccinated and control animals developed suppurative lesions at the site of challenge. Two control animals died with severe haemolytic anaemia within 24 hour after challenge. Surviving controls had multiple abscesses. Out of the 24 vaccinated animals only three had lesions which were single; two of these lesions were sterile for bacteria. The authors concluded that an immune response to the exotoxin played a significant role in resistance. Similar results were obtained by Burrell (1978b) who found that sheep receiving toxoid with adjuvant or toxoid plus formalin inactivated cells incorporated in adjuvant had better protection than animals which received washed formalin inactivated cells with adjuvant.

Anderson and Nairn (1982) Vaccinated goats with a toxoid and challenged them by abraded skin method. Three months after challenge, only three out of 20 vaccinated compared to 10 out of 10 controls had abscesses at slaughter.
Brogden, et al. (1984) examined whole cells and cell wall for their immunizing properties in lambs. They found that there were significantly fewer abscesses and fewer organisms in vaccinated animals. The lambs inoculated with the cell wall preparation had fewer lesions compared to these inoculated with whole cells.

Brown et al. (1986) vaccinated 10 lambs with a toxoid mixed with Freund’s incomplete adjuvant and challenged them by intradermal inoculation. Thirteen weeks later, 8 out of 10 vaccinated lambs were free of disseminated lesions at post mortem compared to four out of five controls, which showed disseminated lesions.

Lea Master et al. (1987) evaluated the efficacy of *C. pseudotuberculosis* vaccine to protect sheep from CLA in controlled challenge exposure experiment. Lambs were vaccinated subcutaneously in the right axillary region with a bacterin. Twenty weeks later, they were challenged subcutaneously at four different sites with $6 \times 10^6$ CFU of *C. pseudotuberculosis* and at week 33 post vaccination, the animals were killed and necropsied. The number of abscesses in each lamb was counted. Results indicated that the vaccine provided immunological protection of lambs against challenge exposure to *C. pseudotuberculosis*. Holstad, Teige and Larsen (1989) used crude filtrated *C. pseudotuberculosis* toxoid and
whole killed organisms, levamisole was included as immunostimulant. Four weeks later, animals were challenged subcutaneously with live bacteria. Abscesses were significantly more common in unvaccinated animals at post mortem two months after challenge. However, all groups developed abscesses at the inoculation site. Brogden et al. (1990) investigated the effect of muramyl dipeptide (MDP) on the immunogenicity of *C. pseudotuberculosis* whole cell vaccine in mice and lambs. Efficacy of vaccination was determined from the survival of mice and appearance of lesions in lambs after intravenous injection of $10^4$ CFU of *C. pseudotuberculosis*. In mice, protection was related to the concentration of whole cells (W.C) in the vaccines. At 50 μg, 100 μg, or 150 μg of W.C, protection was good (78.8%). At 10 μg or 25 μg of W.C, protection was considerably less (54.7%). At low W.C. concentration, protection significantly decreased to 32%. Eggleton et al. (1991a) vaccinated groups of sheep with vaccines of *C. pseudotuberculosis* toxoid combined in varying amounts with five clostridial antigens. These groups were infected at 1, 6 and 12 months after vaccination with pus from ovine lymph nodes which showed infection with *C. pseudotuberculosis*. Three months after challenge, resistance was assessed by slaughter and inspection of the sheep for CLA lesions. They found a significant reduction of lesions in the immunized
sheep compared with control ones. Also they found a positive correlation between amount of *C.pseudotuberculosis* toxoid administered and degree of protection obtained. Eggleton *et al.* (1991b) immunized sheep with *C.pseudotuberculosis* vaccines prepared from toxoid solely or from toxoid with addition of formalin-killed cells of *C.pseudotuberculosis*. Sheep were challenged 6 months after vaccination with caseous lymphadenitis pus through skin incisions. Resistance was assessed three months later by slaughter and inspection of sheep for lesions of CLA. Immunized sheep were protected and there was reduction in the number of sheep exhibiting CLA lesions compared to control animals. Some of these animals were free of lesions; few showed lesions which were smaller in size and lesser in number compared to control non-vaccinated sheep. They found that there was no improvement in protection by inclusion of whole cells of *C.pseudotuberculosis*. Simon-Valenica *et al.* (1992) prepared three vaccines from defatted whole cells of *C.pseudotuberculosis* with different adjuvants: trehalose 6-6 diester (Vaccine A) dextran sulfate (Vaccine B) and Al (OH)3 with saponin (Vaccine C). The fourth vaccine was a toxoid prepared from *C.pseudotuberculosis* exotoxin in water-in-oil emulsion. Four groups of 10 four-week-old mice were given two subcutaneous injections of one of the vaccines at four-week interval, then challenged
interavenously 2 weeks later with virulent *C.pseudotuberculosis*. The best but not complete protection was given by vaccine B.

Uysal *et al.* (1993) tried BCG in inducing immunity for lambs against *C.pseudotuberculosis*. Thirty healthy one-month old lambs with negative intra-dermally (i/d) tuberculin test were used. Twenty were injected i/d with 0.1 ml BCG vaccine. Tuberculin tests, 1 and 2 months after vaccination showed increased skin reactions (skins thickness increased 2-5 times), tube agglutination tests with *corynebacterium* antigen were also positive two months after BCG vaccination. The vaccinated and control sheep were then challenged by i/d injection using *C.pseudotuberculosis* in the prescapular region. Of the 10 controls; two died in one week, while abscesses developed at the injection site, the vaccinated animals remained healthy. Alive sheep gave positive tube agglutination tests after challenge. Pepin *et al.* (1993) determined the efficacy of acquired immunity in controlling CLA and the effect of primary infection on subsequent exposure. Adult seronegative ewes were inoculated with streptomycin-resistant strain 19R *C.pseudotuberculosis* in the right ear. This protocol indicated that the primary infection with at least $10^7$ viable bacteria, induced strong protection against subsequent challenge exposure whereas immune-naïve ewes developed numerous pyogranulomas in the right ear, in lymph nodes.
draining the inoculation site, and in the lungs. However, ewes with primary infection remained carriers of the organism as a result of primary inoculation.

A ribosomal preparation of *C. pseudotuberculosis* for immunization against CLA in Sudan has been examined (Suleiman, 1995). The ribosomal preparation was diluted to an appropriate concentrations with phosphate buffer, filter sterilized, and inoculated s/c into 8 week old male and female Swiss mice with or without Freund’s incomplete adjuvants (1:1) Saponin at an amount of 20μg/mouse was also used as an immunostimulant. Control groups without immunization which were inoculated with adjuvants alone were included. All mice were challenged s/c with 2.4 X10^2 CFU *C. pseudotuberculosis* 10 days after initial inoculation. Their results showed that at low concentration of single dose of 100μl ribosomal preparation, saponin did not enhance any protection, whereas with Freund’s incomplete adjuvant (FIA) there was 25% protection. When animals were immunized with 200μl dose administered with FIA and saponin, protection was 75% and 80% respectively compared to 30% when the same dose was administered without an adjuvant, and controls showed no protection. Mice which received one dose of 200 μl showed 70% and 60% protection for the ribosomal preparation administered with saponin and FIA, respectively,
whereas mice receiving an initial immunization dose (200 μl) followed by a booster of an equal amount, after one week showed 100% and 60% protection for the preparation with saponin and FIA, respectively. All control mice showed no protection. Animals immunized with ribosomes in saponin showed 100% protection when challenged via the s/c and i/v routes, while those receiving the preparation in FIA showed 60% and 50% protection for the s/c and i/v routes, respectively. They stated that the ribosomal preparation from *C.pseudotuberculosis* protected immunized mice against challenge by the same organism. The degree of protection depended on the dose. The highest levels were obtained when 1-2 weeks immunization schedules was followed and it was significantly enhanced by incorporation in adjuvants. They found that protection was better when saponin was used in comparison to Ferund’s incomplete adjuvant.

In a field investigation, Stanford *et al.* (1998) found 50-94% of sheep had antibodies to *C.pseudotuberculosis* prior to initial vaccination. To assess the efficacy and impact of vaccination with two commercial (Glanvac-6 and Case- Vac) and one experimental whole cell vaccine containing the synthetic adjuvant muramyl dipeptide -sn- glyceryl-dipalmitoyl (WC+MDP-GDP) CLA vaccines, a series of three field trials in 3241 ewes and lambs were conducted in affected flocks from 1992-1996.
Overall, agglutinating antibody titers to *C. pseudotuberculosis* in lambs vaccinated with WC+MDP-GDP and Case-Vac, remained significantly elevated above unvaccinated control lambs for 12 months after the vaccination. Lambs vaccinated with the WC+MDP-GDP maintained higher titres than those vaccinated with Case-Vac for 6-12 months after vaccination. Agglutinating antibody titres form lambs vaccinated with Glanvac did not differ from those of controls for months after vaccination. The number of injection site reactions was elevated in lambs vaccinated with Glanvac compared with those vaccinated with WC+MDP-GDP, but size of infection site reactions did not significantly differ. Sheep vaccinated with WC+MDP-GDP also had a reduced incidence of CLA abscesses, although confirmation of the presence of *C. pseudotuberculosis* was only successful in 5 cases.

Hodgson *et al.* (1999) evaluated the efficacy of CLA vaccine formulated using genetically inactivated PLD in sheep. The vaccine protected 44% of sheep against *C. pseudotuberculosis* challenge compared with 95% protection offered by the formalin inactivated preparation.

Chaplin *et al.* (1999) performed a large-scale vaccination trial in sheep to investigate whether an antigen targeted by CTLA-4 enhanced and accelerated the humoral immune response. Vaccination with genetically
detoxified phospholipase D has been shown to be effective, at least partially, against *C. pseudotuberculosis* infection. CTLA-4 bind to B7 on antigen-presenting cells and thus was used to direct the fusion antigens to site of immune induction. They demonstrated that targeting phospholipase D as a CLTA-4 fusion protein significantly enhanced the speed, magnitude and longevity of the antibody response compared to that obtained with DNA encoding phospholipase D. While all groups of sheep vaccinated with DNA encoding phospholipase D were afforded better protection against an experimental challenge with *C. pseudotuberculosis* than those immunized with an irrelevant plasmid or those left unimmunized, the best protection was provided by the targeted DNA vaccine.

Abdel Wahab (2000) in an immunization study prepared two vaccines, cell wall (C.W) lysate and C.W lysate + toxoid. The two vaccines were incorporated in saponin. Three doses of each vaccine with one-month interval were injected s/c into two groups of goats of five animals in each. The third group was left as unvaccinated control. Three weeks after the last vaccine dose all animals were challenged i/v by $1.2 \times 10^5$ CFU/ml of *C. pseudotuberculosis* C P 41N. Then one month after challenge all animals were slaughtered and examined for CLA lesions.
There was significant reduction (P<0.01) in lesions in animals vaccinated with C.W lysate and C.W lysate + toxoid compared with the controls. On the other hand there was significant reduction (P<0.05) in the number of lesions in animals vaccinated with C.W lysate + toxoid compared with animals vaccinated with C.W lysate alone. After challenge, two animals (40%) died from both C.W lysate vaccinated group and the control group. Whereas there was no death among animals vaccinated with C.W lysate + toxoid. In this latter group there were only two animals with lesions (40%), whereas in the C.W lysate vaccinated and control animals lesions were found in all animals (100%). The percentage of lesions in internal organs (lungs, livers, spleens, kidneys, pericardia, omenta, mediastinal and mesenteric lymph nodes) was 20%, 30%, and 57.5% for C.W lysate + toxoid, C.W lysate vaccinated animals and controls, respectively.

Fontaine et al. (2006) using a virulent isolates of *C.pseudotuberculosis* from United Kingdom (UK), an ovine experimental model of CLA was developed, in which the manifestation of disease was equivalent to the naturally observed infections in UK. Subsequently, the capacity of several experimental vaccines to protect against experimental challenge was determined. Sheep were immunised with a recombinant derivative of phospholipase D, derived from the virulent UK isolate, a formalin-killed
bacterin of the same strain, or a bacterin supplemented with recombinant phospholipase D. Following homologous experimental challenge, the phospholipase D and bacterin vaccines were observed to confer statistically significant protection against infection, and appeared to restrict dissemination of challenge bacteria beyond the inoculation site in the majority of animals. More importantly, the combined vaccine succeeded in providing absolute protection against infection, whereby challenge bacteria were eradicated from all vaccinates. In addition to the experimental vaccines, a commercially available CLA vaccine, unlicensed for use in the European Union, was assessed for its capacity to protect against heterologous challenge. The vaccine conferred significant protection, although the dissemination of infection beyond the inoculation site was not restricted as it had been with the previous vaccines. However, no animals immunised with this vaccine manifested infection within the lungs; thus, a potentially important route of disease transmission was eliminated.

Braga (2007) evaluated the immune potential of different doses of cell wall and toxin components of *C. pseudotuberculosis* from alpaca origin, 12 adult alpacas were allotted at random to four groups, and were s/c inoculated in the left flank with vaccines composed of low and high doses of bacterial crude antigens, cell wall: 250 and 500 mg/ml and toxin:133 and 265 mg/ml,
respectively. The vaccines were supplemented with 20 mg/ml of muramyl dipeptide as adjuvant. Three alpacas were inoculated with adjuvant alone as a control. After three weeks, immunized and naive alpacas were challenged intradermally in the right flank with $1 \times 10^6$ colony forming units (CFU) of *C. pseudotuberculosis*. The alpacas were sacrificed at days 28, 58 and 112 after inoculation, and the degree of protection induced by vaccines was demonstrated by the absence of abscesses and/or bacteria. The alpacas vaccinated with high doses of the toxin, did not show abscesses. In contrast, the alpacas vaccinated with a low dose of toxin showed abscesses at the inoculation site, regional, and renal lymph nodes. The alpacas vaccinated with cell wall showed a lesser degree of protection than the other groups with superficial and internal abscesses. The control alpacas had persistent fever and abscesses at the inoculation site, regional, and internal lymph nodes. In addition, a robust and early humoral response was observed in all vaccinated alpacas after challenge, lasting at least three months.

In a study Moura-Costa *et al.* (2008) used four different antigenic extracts obtained from the attenuated strain T1, which was isolated in the state of Bahia (Brazil). Forty-four Canindé breed goats were divided in five groups, each received a different antigen solution and saline buffer only for the control group. The humoral response was monitored through the
identification of specific IgG by indirect ELISA and Western Blotting, and the production of IFN-γ was followed in order to observe the activation of cellular response. After 12 weeks of antigen inoculation, the animals were challenged with $2 \times 10^5$ CFU of a wild strain of *C. pseudotuberculosis*, also isolated in Bahia, and necropsy was performed on all animals 12 weeks afterwards. It was observed that the attenuated bacteria gave a protection of 33.3%, and a humoral response was elicited. Animals inoculated with antigen associated with Freund's incomplete adjuvant and oligodeoxynucleotide containing unmethylated CpG dinucleotides (CpG ODN) showed a strong humoral response, but this inoculation could not prevent the spread of challenge bacteria in the majority of animals.

1.2. Methods of cultivation of *C. pseudotuberculosis* for mass production:

Different methods for mass cultivation were tried using solid agar and liquid media either in a batch or continuous culture. The variability of the strains and the amount of the CFU per dose were considered as the most critical factor in determination of a vaccine efficacy.

The theory of continuous bacterial cultivation in liquid media was first discussed by Monod (1942) and Novick and Szilard (1950). Based on that, microbial growth in a continuous culture occurs at a constant rate and at constant environment. factors such as a pH value, concentration of
nutrients, metabolic products and oxygen are constant. Some of the early workers (Golle, 1953; Fin and Wilson, 1954) disagreed with the concepts drawn by Monod (1942) and Novick and Szilard (1950), but many others considered the concepts had constructed the basis of a correct theoretical treatment for bacterial cultivation in static and continuous systems (Herbert et al., 1956)

1.2.1. Batch culture:

Batch culture is the growth of bacteria in a system where no fresh nutrients are added, nor the metabolic products are removed during the process of growth, which causes progressive changes in the medium component that no further growth could be supported. The characteristic sequence of changes are referred to as the "growth cycle" (Monod, 1949; Pirt, 1975).

When environment remain favorable, growth will continue until one of the essential substrates in the medium is depleted, and it is called the growth limiting substrate if all other nutrients are in excess.

The specific growth rate (the rate of increase / unit) of microorganisms is dependant on the concentration of the growth limiting substrate according to the empirical equation of Monod (1942, 1949):
\[ \mu = \mu_{\text{max}} \left( \frac{S}{ks + S} \right) \]

Where \( \mu \) is the specific growth rate, \( \mu_{\text{max}} \) is the maximum specific growth rate, \( S \) is the concentration of the growth limiting substrate and \( ks \) is a constant, numerically equal to the substrate concentration at which \( \mu = \frac{1}{2} \mu_{\text{max}} \).

Monod (1942) also demonstrated the relationship between growth rate (\( dx \)) and the substrate utilization (\( ds \)) in the following equation:

\[
\frac{dx}{ds} : \frac{\text{weight of bacteria formed}}{\text{weight of substrate consumed}} = y
\]

Where \( y \): is the growth yield constant

If the three growth constants \( \mu_{\text{max}}, ks \) and \( y \) are known, the above two equations will provide a complete description of the growth cycle in a batch culture (Monod, 1942).

**1.2.2. Continuous cultures:**

Continuous culture, consists of a culture vessel to which fresh nutrient medium is constantly supplied, instantly mixed with the culture thus ensuring good homogeneity. The volume of culture in maintained constant by an overflow system. Factors such as pH value, concentration of nutrients, metabolic products and oxygen, which inevitably change during the growth cycle of a batch culture, are maintained constant in a continuous
culture. Initially all continuous systems start as batch culture and the continuous flow of fresh medium will provide the culture with additional growth limiting substrate, allowing the growth rate of the organism to be controlled by the rate of addition fresh medium. The organism is simultaneously harvested from the vessel by the overflow device, and the population will be maintained by multiplication provided that the rate at which the culture is diluted by addition of fresh medium does not exceed a certain critical value.

The most commonly used continuous culture system is the flow-controlled device, usually referred to as the chemostat (Herbert et al., 1956). In this system the volume of the medium is V, the dilution rate D of the culture is \( D = \frac{F}{V} \), where \( F \) is the flow rate (volume of the fresh medium added to the culture vessel / unit time) and the bacterial growth follows an experimental pattern, and the growth rate is expressed by the formula:

\[
\frac{dx}{dt} = \mu x
\]

Where \( x \) is the cell mass (g/l), \( \mu \) is the specific growth rate and \( t \) is the time. The change in the concentration of bacteria in the culture is:

Change = growth – output

Since growth is \( \mu x \) and output is \( Dx \) then:
\[
\frac{dx}{dt} = \mu x - Dx \quad \text{or} \quad \frac{dx}{dt} = x(\mu - D)
\]

The culture will be in steady state wherever \( \mu \) is equivalent to \( D \), (i.e.) the bacterial mass added to the system equals to that removed from it. There will be no change in status and the bacterial concentration of the organism will remain constant with time. When \( \mu > D \), \( \frac{dx}{dt} \) is positive and the concentration of the organism will increase, when \( D > \mu \), \( \frac{dx}{dt} \) is negative, and the concentration of the bacteria will decrease and the culture will be washed out (Herbert et al., 1956). Figure 1 shows the variation of steady-state concentration, the mean generation time of bacteria and the substrate in continuous culture with dilution rate variation.

1.2.2.1. Types of continuous culture:

According to the operating principles two types of culture apparatus are described:

1.2.2.1.1. Turbidostat:
The operating principle in this type is the maintenance of a constant microbial density (constant culture absorbance). In this case the biomass density is first fixed and the dilution rate regulates itself to the required biomass level. It was found that turbidostat continuous culture is very difficult to stabilize (Bryson, 1952).

1.2.2.1.2. Chemostat:
The organism in this type, can be cultured continuously at a specific growth rate less than \( \mu \text{max} \) (Novick and Szilard, 1950). The culture is maintained at a fixed volume to which the medium is pumped at a constant rate and the culture is removed from the chemostat at an equal rate.
Fig. 1: Steady-state relationship in continuous culture (Herbert et al., 1956).
1.2.2.1.3. Application:

According to Golle (1953) and Herbert et al. (1956), the advantages of continuous cultivation technique of microorganisms to batch culture are:

- Increase of production, continuous culture may usually be expected according to Golle (1953) and Herbert et al. (1956) to show a five to ten fold increase in output as compared with a batch culture.

- The system is more adaptable to automation.

- A uniform product is obtained.

- Steady load on the utility source is possible with no peak loads.

- More economic to industrial microbiology.

- Valuable research tool

Difficulty in maintaining sterility during long runs that is frequently raised against the continuous system. When assembling the different parts of the fermenter and by applying strict sterile measures contamination could be controlled and runs of several months are now routine in IBT bioreactor (Böhnel, 1986; Roth, 1986; Babiker, 1991).

Mutation, is another difficulty of continuous culture but is a rare event arising singly, and a single organism with a generation time T has a probability of $1 - e^{\Delta t}$ of being washed out of the culture vessel before it divides once. At this time a considerable number of mutants arising in a continuous culture will be removed before they have progeny, while in batch culture all will remain (Herbert et al., 1956). Böhnel (1986) described the continuous cultivation of bacteria as state of long-term repeated division of bacterial cells, which leads ultimately to selection rather than to mutation.
### 1.2.3. Methods of concentration of bacteria:

Different methods like filtration, centrifugation precipitation and foam separation have been used for antigen purification or concentration and separation of the organisms from cultures, the most practical one is filtration.

#### 1.2.3.1. Filtration:

Filtration as a mean of bacterial harvesting and concentration has found its application in improvement of vaccine production. It is considered as the least laborious, fast and most economical method for concentration.

#### 1.2.3.1.1. Cross flow filtration:

Cross flow filtration is used to produce pure liquids, for concentration of suspensions, and recovery of valuable process liquid (Hood, 1990). Cross flow filtration either simultaneously, during the fermentation processes, or alternatively as downstream processing at the end of fermentation (Grabosch, 1987). Tangential cross flow filtration is a pressure driven membrane moderated filtration process in which the particles to be separated flow parallel to the membrane surface in a tangential direction (Hood, 1990; Grabosch, 1987). The solvent with a small filterable particles pass through the filter due to pressure gradient, which leads to the concentration of non-filterable material in flowing solution (Strohmaier, 1967). The rapid strong and continuous flow of the suspension to be filtered in across flow filtration system, reduce significantly the building of secondary membrane, however it will not prevent its formation completely (Michales, 1968; Strathmann, 1981; Rähse, 1985). Hence, this filtration technique is superior to the other types (Alouf and Raynoud, 1970; Gram and Lai, 1971). Tangential filtration , especially the cross flow technique in hollow fibers is mostly
done in recirculation systems, which employ pumps as driving forces (Gangeni et al., 1977; Klein, 1981).

1.2.3.2. **Types and structures of filters:**

Strathmann (1981) and Böhnel (1986) described a variety of synthetic filters, which differ in their membrane structure, material and application. The basic required qualities of any filter are the thermal, chemical and biological stability together with the other necessary physical characteristics (Browne, 1942; Michales, 1968; Shiele and Alt, 1978).

1.2.3.2.1. **Membrane filters:**

Membrane filters are integral structures made of thin highly purified porous sheets of different materials; their properties depend on the manufacturers. Different uniform pore size filters ranging between 0.22-5.0 μm in diameter of inert cellulose esters used for both air (Timothy and Raymond, 1984) and liquids filtration with high flow rates are available (Guadfrin and Sabatier, 1978).

1.2.3.2.2. **Hollow fiber filters:**

Hollow fiber filters are made of very tightly and parallely arranged hollow fibers having a diameter of 0.2 - 1.1 mm (Saier, 1977) and are supported with a metal, plastic or glass framework. Different hollow fibers of different permeabilities are used for the separation of toxins and substances of low molecular weight.
Chapter Two
Materials and Methods

2.1. Field survey:
Two different categories of sheep were surveyed for CLA, the first one is live ones at animal markets, and the second is the carcasses of slaughtered animals during meat inspection.

2.1.1. Examination of live animals:
A total of 6560 sheep and 4450 goats were examined clinically for the presence of superficial abscesses at the local animal markets in and around Nyala town, South Darfur state, Western Sudan during the period from February 2004 to May 2005. Animals were brought to those markets from different parts of the state, and thus representing the animals in the state. A total of 54(0.82%) and 34(0.76%) samples of lymph nodes or abscesses were collected from sheep and goats, respectively.

2.1.2. Examination of slaughtered animals at meat inspection:
A total of 3324 sheep and 184 goats were inspected at post-mortem examination for presence of abscesses at Nyala slaughterhouse. Eighty nine (2.8%) and 6(3.3%) affected lymph nodes or abscesses were collected from
sheep and goats, respectively.

2.1.3. Sample collection:

Live animals were examined carefully for enlarged superficial lymph nodes. The outer surrounding skin of these lymph nodes was shaved and disinfected with alcohol. Pus was aspirated using sterile needles and syringes.

Lymph nodes were collected from animals slaughtered at Nyala slaughterhouse during the meat inspection, subcutaneous abscesses or enlarged lymph nodes samples were removed carefully from the carcass and put separately in sterile plastic bags, labeled and taken immediately in a thermos flask containing ice to the Laboratory for culturing.

2.1.4. Staining methods:

2.1.4.1. Preparation of smear:

Direct smears were prepared from puss samples, liquid cultures or emulsified colonies on clean dry glass slides. The smears were allowed to dry in air and then fixed by gentle flaming and stained with the Gram stain.
2.1.5. Culture samples and identification of isolates:

2.1.5.1. Culture Media:

2.1.5.1.1. Solid media:

- Blood agar base No. 2 (Oxoid CM55):

- Brain heart infusion agar (Oxoid CM375)

- Nutrient agar (Oxoid CM3, R2)

- Urea agar base (Oxoid CM53, R3)

- Serum agar slant

- Dorset egg medium (Oxoid PM5)

2.1.5.1.2. Liquid media:

- Nutrient broth (Oxoid CM1, R1)

- Brain heart infusion broth (Oxoid CM225)

- Robertson’s cooked meat medium (Cowan, 1974)

- Nitrate broth (Cowan, 1993)

- Peptone water sugars (Cowan, 1993)
2.1.5.2. Reagents:

- Hydrogen peroxide
- Nitrate test reagents

2.1.5.3. Solutions:

- Normal saline (0.85%):
- Phosphate buffered saline solution (PBSS)

2.1.6. Biochemical methods:

2.1.6.1. Catalase test:

A drop of 3% aqueous solution of hydrogen peroxide was put on a clean microscope slide. A colony of test culture, on nutrient agar, was placed on the hydrogen peroxide drop. The test was considered positive if gas bubbles evolved on the surface of the culture material.

2.1.6.2. Urease activity:

Urease activity was shown by alkali production (ammonia) from urea split by the test organism. Heavy inoculums was transferred to the urea agar slope and incubated at 37°C and examined daily. A positive test was indicated by pink or red colour; negative results were not considered before the end of 7 days.
2.1.6.3. Nitrate reduction:

To test the ability of the organism to reduce nitrate to nitrite, the test culture was inoculated in nitrate broth, and incubated at 37°C for two days. One ml of solution A (sulphanilic acid) was added to the test culture followed by 1 ml of solution B (α- naphtylamine). A positive reaction was indicated by development of red colour. If the result was negative zinc dust was added. A red colour indicated the presence of nitrate which was reduced by zinc to nitrite.

2.1.6.4. Carbohydrates fermentation:

Peptone water and nutrient broth sugars were inoculated with the organism under test and examined daily for seven days for acid production.

2.1.7. Blood and serum collection:

Blood and serum used to enrich media were collected by veni-puncture of the jugular vein of a healthy sheep. Blood was collected in sterile containers, defibrinated with sterile glass beads (1.5-2 mm. in diameter) and used for preparation of blood agar and sheep erythrocytes suspension for haemolysis tests. Blood needed for serum preparation was allowed to clot at a room temperature for one hour. By using a sterile glass rod the clot was separated
from the walls of the containers, which were then transferred to a refrigerator at 4℃ and left overnight. Serum was pipetted into tubes and centrifuged at 3000, r.p.m for 10 minutes and the clear straw-cloured fluid was dispensed in 10 ml amounts, inactivated in water-bath at 56 ℃ for 10 minutes then stored frozen at –20℃ until needed. The serum was used for serological tests was collected from vaccinated or control animals.

2.1.8. Sterilisation:

Glassware such as petri-dishes, tubes, flasks, graduated pipettes and glass beads were sterilised in a hot air oven at 160℃ for 90 minutes.

Capped bottles, swabs, solutions and all media except sugars were sterilised at 110℃ for 5 min. Cotton stoppered tubes were wrapped in yellow paper, and sterilized in the autoclave at 121℃ (15 lb/ square inch) for 15 minutes.

2.1.9. Isolation of *C. pseudotuberculosis*:

When abscesses or lymph nodes were brought to the laboratory, they were cut open by means of sterile scalpels after searing the surface with hot spatulas. From the periphery of the lesions, a loopful of pus was streaked onto blood agar plates. The plates were incubated microerophilically, in a desicccator, under 10% CO₂ tension at 37 ℃ for 48 hours.
2.1.10. Identification of isolates:

From a single colony on blood agar, a thin smear was made on a clean glass slide. This was air dried, fixed by heat, stained by the Gram’s method and examined under the oil immersion lens for the characteristic short coccobacilli. The isolates were tested for catalase and urease activity and nitrate reduction. Purification was accomplished by subsequent subcultures on blood agar plates to obtain pure cultures.

2.1.11. Preservation of isolates:

To preserve isolates of *C. pseudotuberculosis*, were inoculated in serum agar slants, Dorset egg slants and cooked meat medium, and incubated for 24 hours at 37°C, then after check for purity, were stored at –40°C.

2.2. Mass Cultivation of *C. pseudotuberculosis* using IBT Bioreactor:

2.2.1. Bioreactor setting up and assembling:

The system is constructed and developed to cope with the conditions in the tropical countries. The bioreactor was used successfully for production of bacterial vaccines (Roth, 1986; Haußer, 1989; Babbiker, 1991; Schaper, 1991; Elbashir, 1993; Sonnenberg, 1993; Rengel, 1993; Suleiman, 2001; Mona El haj, 2002). In addition to some vaccines against some viral diseases (NiBlein, 1993; Ullrich, 1994).

The IBT bioreactor is a closed system for medium sterilization, bacterial
culturing and harvesting. The internal positive pressure made by a circulation pump plays the main role in protection of the system against contamination (Figure 2).

The bioreactor consisted of the following functional parts:

- Bioreactor control circuit.
- Fermenter tank.
- Aeration system.
- Medium sterilization and supply
- Air filters.
- Sampling system.
- Harvest and inactivation unit.

The other basic, replaceable and autoclavable materials of the IBT bioreactor were:

- Silicone tubes and stoppers of different sizes
- Glassware.
- Stainless steel tubes of different sizes.
- Stainless steel male and female connectors (Swagelock).
- Polypropylene connectors (T and Y shapes).
- PTFE ring septa coated magnetic rods, silicone ring septa.
Fig. 2: IBT bioreactor set-up for production of *C. pseudotuberculosis*
2.2.2. Propagation of *C.pseudotuberculosis*:

2.2.2.1. Bacteria:

A local strain of *C.pseudotuberculosis* strain CP 41N isolated from sheep was used as a seed for inoculation in bioreactor. This strain was selected because of its high growth yield and increased haemolytic titre (Abdel Wahab, 2000).

2.2.2.2. Preparation of seed and inoculum:

Lyophilized *C.pseudotuberculosis* strain Cp41N was reconstituted in nutrient broth, incubated at 37 °C for 24 hours then injected I/M into a rabbit. The rabbit died after 12-18 hours, samples from liver and site of injection were cultured onto blood agar, after 24 hours of incubation at 37 °C, from the colonies of *C. pseudotuberculosis* that appeared one colony was taken into brain heart infusion broth with 0.1% Tween 80 (BHIB+T). One milliliter of the broth was inoculated into 50 ml of BHIB+T, incubated at 37 °C for 24 hours. Growth could be detected by turbidity. Smears were made from the broth, stained by Gram's stain to check purity. This inoculum was used for inoculation the fermenter.

2.2.2.3. Media used:

Burrell’s liquid media (BLM) was used, which contained:

- Proteose peptone 10g
- Meat extract 5g
- Sodium chloride 5g
- Yeast extract 1g
- Glucose 2g

2.2.2.4. Production of C. pseudotuberculosis culture:

The fermenter parts were assembled after autoclaving. A temperature of 31º C, pH of 8.0 and dilution rate at 0.02 h⁻¹ were adjusted. The volume of the media in the system was 500 ml. The system circulated, samples were taken daily and smears were stained with Gram's stain to check purity. The harvested fermenter broth was collected in 0.3% formol saline (Böhnel, 1999 and Suleiman, 2001).

2.2.2.5. Filtration of harvested fermenter broth:

For separation of cells and toxins polypropylene hallow fiber filters (FRESENIUS, St. Augustin) SPS900 (MWCO/1000 kDa) and SPS600 (MWCO/100 kDa) were used. Two phases of filtration were done to obtain purified and concentrated products from the formalinized fermenter broth, which could be carried out either simultaneously or separately. In phase 1 the bacterial cells were separated from the medium which contains remaining medium ingredients, toxoid and metabolic products. In phase 2, further filtration to purify and concentrate toxoid by discarding low molecular weight proteins, the rest of medium components and water.
Fig. 3: An illustration for filtration system set-up (Böhnel, 1999).
2.2.3. Viable cell count:

The viable cell count of *C.pseudotuberculosis* in samples from the bioreactor was determined according to Miles and Misra (1938), it was found to be $1.5 \times 10^8$ CFU/ml.

2.2.4. Components of different vaccines:

Five types of vaccines were prepared by different combinations from three components; whole cell, cell wall lysate and toxoid.

2.2.4.1. Whole cell:

After collection and filtration of harvested fermenter broth, separated cells were washed with distilled water and stored at 4º C as whole cell concentrate.

2.2.4.2. Cell wall lysate:

Whole cell concentrate was twice with acetone and twice with diethyl ether. The cells were then disrupted either by shaking with glass beads by means of an electronic shaker (Edmond, Germany) and/ or by sonicator (MSE, England). Trypsin 1µg/ml was added and the suspension was incubated at 37º C for six hours. By centrifugation, the cell wall lysate was pelleted, and then washed three times in distilled water. The lysate was examined microscopically after staining by Gram’s to detect intact cells.

2.2.4.3. Toxoid:

From the formalinized fermenter broth harvest toxoid was collected by filtration,
the protein content was determined for the toxoid, then incubated at 37º C for 45 days during which the formalin concentration of 40g/l which corresponding 0.3% formalin was maintained. After that the toxoid was stored at 4º C

2.2.5. Determination of protein content for vaccines:

The protein content was estimated according to Lowry et al. (1951) method which depends on detection of tyrosine content that is constant in many proteins.

2.2.5.1. Reagents:

- N. Na OH containing 2% anhydrous Na₂Co₃.
- 2% sodium /potassium tartarate solution.
- 1% Cu So₄ solution.
- Bovine serum albumin (BSA), 1mg/stock solution.
- Folin Ciocalteu phenol reagent (BDH) diluted 2:5 distilled water.

All reagents were prepared in distilled water. Lowry solution was prepared by adding 50 ml of 0.1 N Na OH containing 2% anhydrous Na₂Co₃ solution A, to 0.5ml of 2% sodium/potassium tartarate solution B, Then 0.5 ml of 1% Cu So₄ was added

2.2.5.2. Procedures:

The bovine serum albumin (BSA) was used as standard protein and was prepared by dissolving 50 mg of BSA in 50 ml volumetric flask to give a
stock solution of 1 mg protein per 1 ml and the following dilutions were made in test tubes, 200 μg/ml, 400 μg/ml, 600 μg/ml 860 μg/ml, 1000 μg/ml. Likewise, in separate test tubes 1/5, 1/10 and 1/20 dilutions of the unknown solutions “whole cell, cell wall lysate and toxoid” were made. From each dilution of the standard protein or the solution under test, 0.1 ml was pipetted separately into fresh test tubes and the volume was brought to 0.4ml by addition of 0.3 distilled water. All tests were made in duplicates. The final concentration of the standard protein were 20, 40, 80, 100 micrograms protein. For blank 0.4 ml of distilled water was put into two test tubes. To the standard and each dilution of the test solutions, 2ml of Lowery solution was added. The tubes were mixed well and incubated at room temperatures for 10 minutes in order to allow protein hydrolysis. Then to each tube, 0.2 ml of Folin Ciocalteu phenol reagent diluted 2:5 with distilled water was added. Test tubes were mixed and immediately kept in the dark for 30 minutes.

Absorbance of each standard and test solution was read in a spectrophotometer (Milton Roy, U.S.A), using 1cm path at 560 nm.

A standard curve was blotted using protein value against absorbance of standard protein. The protein value of the toxin was estimated from the standard curve.
2.3. Vaccination trials:

2.3.1. Experimental animals:

Forty two sheep of local types at ages from six months to one year old, weighing (15-30kg) and seronegative for *C.pseudotuberculosis* by the bacterial agglutination test, were bought from the local market. The animals were divided at random into 6 groups, 7 sheep each. They were kept in pens, and dewormed. Feed and water were provided *ad libitum* and observed for adverse clinical signs for five weeks before vaccination.

2.3.2. Composition of vaccines:

2.3.2.1. Types of the vaccine:

Five types of vaccines were prepared as follows:

(i) Whole cell vaccine that a dose of 0.5 ml contained 8 mg of dry weight.

(ii) Cell wall vaccine that a dose of 0.5 ml contained 3 mg of protein.

(iii) Toxoid vaccine that a dose of 0.5 ml contained 4 mg of protein.

(iv) Cell wall and toxoid vaccine of which a dose of 1 ml composed of cell wall (3 mg protein) + toxoid (4 mg protein).

(v) Whole cell and toxoid vaccine of which a dose of 1 ml composed of whole cell (0.5 ml/8 mg dry weight) + toxoid (0.5 ml/4 mg protein).
2.3.2.2. Adjuvant used:

The saponin and aluminum hydroxide (Al (OH)$_3$) were incorporated into each vaccine at a dose rate of 1mg/1ml each.

2.3.2.3. Vaccination protocols:

The five groups of animal were administered the first dose subcutaneously at the shoulder region as fellow:

- Each animal in the first group was injected with 0.5 ml dose of the whole cell (WC) vaccine.
- In the second group with 0.5 ml dose of the cell wall (CW) vaccine.
- In the third group with 0.5 ml dose of the toxoid (T) vaccine.
- In the fourth group with 1 ml dose of the cell wall and toxoid (CW+T) vaccine.
- In the fifth group with 1 ml dose of the whole cell and toxoid (WC +T) vaccine and the sixth group was left unvaccinated control.

After four weeks a booster dose of each vaccine was injected similarly into each animal in the five groups.

2.3.3. Challenge of vaccinated animals:

2.3.3.1. Preparation of the challenge doses:

*C.pseudotuberculosis* strain CP41N was cultured on blood agar, incubated at 37°C for 48 hours; then one colony was transferred with a sterile wire loop to the brain
heart infusion broth containing 0.1% Tween 80 and incubated at 37°C for 24 hours. Ten-fold serial dilutions of the culture were made in normal saline and with a pipette, 0.1 ml amounts were cultured from dilutions $10^{-6}$, $10^{-7}$ and $10^{-8}$ onto blood agar plates in duplicates. After culture the plates were dried in the incubator for one hour, and then incubated at 37°C for 48 hours. Colony-forming units (CFU) from each plate were counted using a colony counter (Gallenkamp, England) means values were determined.

Hence a challenge dose for each animal was determined to be $5.1 \times 10^8$ CFU of *C.pseudotuberculosis* strain CP41 N.

2.3.3.2. Inoculation of challenge doses:

One month after the booster dose of each vaccine, all sheep of the six groups were challenged with the determined challenge dose, each administered subcutaneously on the left side of the neck of each animal.

2.3.4. Serology:

2.3.4.1. Bacterial agglutination test (BAT):

2.3.4.1.1. Preparation of whole cell antigen:

*C.pseudotuberculosis* strain CP41N was cultured at 37°C onto brain heart infusion (BHI) agar containing 0.1% sorbitan mono-oleate. The 24- hours growth was transferred to BHI broth containing 0.1% sorbitan mono-oleate and incubated with
constant stirring for six hours at 37°C. The culture was centrifuged at 3000 rpm for 15 minutes. The packed bacterial cells were washed two times in PBSS at pH 7.2 containing 1.0% sorbitan mono-oleate and 0.3% formalin, and then suspended in PBSS at the same pH containing 1.0 % sorbitan mono-oleate (Keskintepe, 1976) and 0.2 % formalin and diluted to 80% transmittance with a spectrophotometer (Milton Roy, U.S.A) at (600 nm) wave length (Lund et al., 1982).

2.3.4.1.2. Test procedure:
Double dilutions of each test serum were made from: $1/5$, $1/10$, $1/20$ to $1/320$ in 0.5 ml PBSS at pH 7.2 with 0.1% sorbitan mono-oleate, 0.5ml of cells suspension were added. The mixture was incubated overnight in a water bath at 37°C and examined visually for agglutination and clearing of the supernatant. Pooled sera from three infected sheep were used as a positive control.

2.3.5. Autopsy of sheep after challenge:
All animals of the six groups were slaughtered four weeks after challenge to evaluate and compare levels of protection, in comparison to non vaccinated control sheep. As CLA is characterized by abscesses formation in lymph nodes draining the part of C. pseudotuberculosis entrance therefore, the challenge site, the main draining lymph nodes and internal organs of carcasses were thoroughly examined for CLA pyogenic lesions or abscesses. Samples from of lesions were collected aseptically for bacteriological examinations.
2.3.6. Calculation of protective capacity of vaccines:

The capacity of vaccines to protect animals against infection when less than 100% of susceptible controls become affected after challenge can be expressed as a protection percentage using this formula:

\[
\text{Percentage protection} = \frac{\% \text{ of infected control} - \% \text{ of infected vaccinates}}{\% \text{ of infected controls}} \times 100
\]

2.3.7. Statistical analysis:

Appearance of abscesses in the control group and groups of animals vaccinated with WC, CW, T, CW + T, and WC + T was evaluated statistically using Analysis of variance (ANOVA).

2.4. Antibiotic Sensitivity Tests:

2.4.1. Strains used:
Sixty six strains of *C. pseudotuberculosis* isolated in the study area, were selected randomly to be tested for antibiotic sensitivity.

2.4.2. Test procedures:
A colony of each *C. pseudotuberculosis* was transferred from blood agar to 5ml nutrient broth and incubated at 37 C for 24 hours. Two drops from this broth were mixed with 5 ml of normal saline and 1 ml of the mixture was spread over DST
agar (Oxoid 0261). The plates were left for 5 minutes before excess fluid was
removed using a sterile pipette. Antimicrobial discs were dispensed on each plate
with sterile forceps. The diameter of inhibition zone around each antimicrobial
disc was measured with calipers in millimeters.

2.5. Polymerase chain reaction (PCR):

2.5.1. DNA extraction:

TNES buffer:

- 100 mM Tris Hcl (pH 7.6)
- 40 mM EDTA
- 50 mM NaCl
- SDS 0.2%

Chromosomal DNA extraction was made in the same manner for all the
selected 28 *C. pseudotuberculosis* strains. A 20 ml 48–72 h culture of each strain
was centrifuged at 4 °C and 6000 rpm for 20 minutes. The supernatant was
discarded and 800 µl of TNES buffer and 10 µl of proteinase K (10mg/ml) were
added to the precipitate which was incubated over night at 37 °C or at 65 °C for 2
hours. An equal volume of phenol/chloroform/isoamyl alcohol was added, mixed
and centrifuged at 6000 rpm for 5 minutes. The upper layer was transferred to a
clean tube, before an equal volume of phenol/chloroform/isoamyl alcohol was
added. The mixture was centrifuged at 6000 rpm for 5 minutes and the upper layer
was transferred to a clean tube. Two volumes of 95% cold ethanol and 1: 10 of sample volume of 3M Sodium acetate were added and the tubes were incubated over night or at –20°C for 2 hours then centrifuged for 10 minutes at 12000 rpm. The supernatant was discarded and 2 ml of 70% ethanol were added. The tubes were then centrifuged for 7 minutes at 12000 rpm and the supernatant was discarded. The previous step was repeated one more time before the supernatant was discarded and the pellet allowed to dry for 15 minutes and100 µl of TE buffer or deionized water was added and stored at -20° C till use.

2.5.2. Concentration of DNA:

The quantity of DNA for every sample was read by Nano Drop spectrophotometer ND- 100, depending on DNA reading either 2.5 or 5 µl was taken for the PCR.

2.5.3. Primers design:

The primers used in this study were targeting the published 16S rRNA gene sequences of *C. pseudotuberculosis* (GenBank). According to Çetinkaya, (2002) the selected primers with a length of approximately 20 bp, an optimal annealing temperature between 50 and 60° C, and a GC-content between 20 till 80%. The resulting forward primer was;

ACCGCACTTTAGTGTTG (E. coli position 183–203)

and the reversed primer had the sequence:
TCTCTACGCCGATCTTGTAT (E. coli position 1019–999)
and the annealing temperature was 55°C. The specificity of the primers was
checked by comparing their sequences to all known sequences in GenBank
(Çetinkaya, 2002).

2.5.4. PCR mixture:
The total reaction volume was 25 µl which contained:
- 5µl of 10 × Vi buffer A (Vivantis, DNA Amplification products)
- 5 µl of 5 mM MgCl2
- 1 µl of 10 mM deoxynucleotide triphosphate (dNTPs) (Vivantis,
  Nucleotides)
- 0.4 µl of Taq DNA polymerase (Vivantis) conc. 5 µl/µl
- 1 µl of 100 mM of each primer (Vivantis technologies Sdn.Bhd.,
  Malaysia)
- 5 or 2.5 µl of template DNA.

2.5.5. PCR conditions:
The PCR was performed in a touchdown thermocycler (Advanced primus
96, Peq lab Biotechnologie, Germany)
Amplification was obtained with 30 cycles following an initial denaturating
step at 94° C for 5 minutes.
Each cycle involved:

- Denaturation at 94° C for 1 minute.
- Annealing at 56° C for 1 minute.
- Synthesis at 72° C for 2 minute.

2.5.6. Detection of PCR product:

By using gel electrophoresis the amplified products for elected strains with both positive wild strain and negative control were detected by ethidium bromide (0.5 mg/ml) staining after electrophoresis at 60–70 V for 1 h in 1.5% agarose gels. An automated photo documentation system (Bio. Doc. Analyza, digital) was used for analysis. PCR products with the molecular size of 815 bp were considered indicative for identification of *C. pseudotuberculosis*. 
Appendix

1. Culturing and identification:

1.1. Culture Media:

1.1.1. Solid media:

1.1.1.1. Blood agar base No. 2 (Oxoid CM55):

Ingredients: g. per 1 liter

- Proteose peptone: 15g
- Liver digest: 2.5g
- Yeast extract: 5g
- Sodium chloride: 5g
- Agar No.3: 12g

pH 7.4 (approx)

Forty grams were suspended in one liter of distilled water, brought to boil to dissolve the ingredients completely, mixed and sterilized by autoclaving at 121°C for 15 min. Then cooled to 45-50°C and 7% defibrinated ovine blood was added, mixed gently and the media was dispensed in sterile petri-dishes in amounts of 15 ml each.
1.1.1.2. Brain heart infusion agar (Oxoid CM375):

Ingredients: g. per 1 liter:

- Calf brain infusion solids 12.5g
- Beef heart infusion solids 5.0g
- Proteose peptone (Oxoid L46) 10.0g
- Sodium chloride 5.0g
- Dextrose 2.0g
- Disodium phosphate 2.5g
- Agar No.1 (Oxoid L11) 10.0g

pH 7.4 (approx.)

Forty-seven grams were suspended in 1 liter of distilled water, boiled to dissolve the ingredients completely, and (0.1% v/v) Sorbitan mono-oleate (Tween 80) was added. The medium was autoclaved at 121°C for 15 minutes then poured in sterile petri-dishes in amounts of 15–20 ml.

1.1.1.3. Nutrient agar (Oxoid CM3, R2):

Ingredients: g. per 1 liter

- Peptone 5.0g
- Yeast extract 5.0g
Lab Lemco powder 1.0g
Sodium chloride 5.0g
Agar No.1 15.0g

An amount of 8.0 g were suspended in 1 liter of distilled water and brought to boil to dissolve completely. The pH was adjusted to 7.4±0.2 and the preparation was sterilized by autoclaving at 121°C for 15 minutes, then cooled to 45-50°C and distributed into sterile petri-dishes in about 20 ml each.

1.1.1.4. Urea agar base (Oxoid CM53, R3):

Ingredients: g. per 1 liter

Peptone (Oxoid 37) 1.000g
Dextrose 1.000g
Na2 HPO4 1.200g
KH2 PO4 0.800g
Sodium chloride 5.000g
Agar No.3 (Oxoid L 13) 15.000g
Phenol red 0.012g
An amount of 2.4 g were suspended in 95 ml of distilled water and brought to boil to dissolve completely and the pH was approximately adjusted to 6.8. The medium was sterilized by autoclaving at 115°C for 20 min., cooled to 50°C and aseptically 5 ml sterile 40% urea solution were added and mixed well. The medium was then distributed in 10 ml amounts into sterile MacCartney bottles and allowed to set in slope position.

1.1.1.5. Serum agar slant:

The medium was prepared according to Cowan (1993) by the addition of 10% sterile horse serum to melted nutrient agar.

1.1.1.6. Dorset egg medium (Oxoid PM5):

Ingredients:

- Fresh egg mixture (yolk + white) 750 ml
- Nutrient broth powder (Oxoid) 250 ml

The eggs were washed thoroughly with soap and water, soaked in 70% alcohol for 30 minutes, then layered on sterile surface. The shell was broken at the narrow end with sterile knife, the contents were let to fall into sterile flask, and then shaken thoroughly to break up the yolk and to produce a homogeneous mixture. The nutrient broth powder was
suspended in 250 ml distilled water, boiled to dissolve completely and then added to the egg mixture and mixed well avoiding bubbles formation. The medium was then distributed in sterile MacCartney bottles in amounts of 5-7 ml each. For sterilization of the medium, the bottles were sloped in the upper part of steamer until the slant has been formed and then left for 1 hour in the steamer. The procedure was repeated for two successive days.

1.1.2. Liquid media:

1.1.2.1. Nutrient broth (Oxoid CM1, R1):

Ingredients: g. per 1 liter

- Peptone: 5.0g
- Yeast extract: 2.0g
- Lab Lemco powder: 1.0g
- Sodium chloride: 5.0g

Thirteen grams were added to one liter of distilled water, and mixed well to dissolve. The pH was adjusted to 7.4±0.2 and the preparation was then distributed in test tubes in 10 ml amounts and sterilized by autocalving at 121°C for 15 min.
1.1.2.2. Brain heart infusion broth (Oxoid CM225):

Ingredients: g. per 1 liter

- Calf brain infusion solids 12.5g
- Beef heart infusion solids 5.0g
- Proteose peptone (Oxoid L46) 10.0g
- Sodium chloride 5.0g
- Dextrose 2.0g
- Disodium phosphate 2.5g

pH 7.4 (approx.)

Thirty-seven grams were added to 1 liter of distilled water and boiled to dissolve the ingredients completely before 0.1% (v/v) Sorbitan mono-oleate (Tween 80) was added to the medium. The medium was then mixed well and distributed into MacCartney or universal bottles and sterilized by autoclaving at 121°C for 15 minutes.

1.1.2.3. Robertson’s cooked meat medium (Cowan, 1974):

- Minced meat 1000g
- 0.05-N NaOH 1000ml

Clean fat free lean meat was minced into small particles and added to the alkali solution Na(OH), mixed well and heated to boiling, simmered
for 20 minutes with frequent stirring, skimmed off any fat and the pH was adjusted to 7.5, strained through gauze, dried, distributed in screw capped bottles and sufficient nutrient broth was added to cover the meat to 2 cm above. The medium was then sterilized by autoclaving at 115°C for 20 minutes.

1.1.2.4. Nitrate broth (Cowan, 1993)

Ingredients: g. per 1 liter

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K NO₃</td>
<td>1 g</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

KNO₃ was dissolved in nutrient broth, distributed into sterile test tubes and sterilized by autoclaving at 115 °C for 20 min.

1.1.2.5. Peptone water sugars (Cowan, 1993):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>900 ml</td>
</tr>
<tr>
<td>Andrade’s indicator</td>
<td>10 ml</td>
</tr>
<tr>
<td>Sugar</td>
<td>10 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

pH 7.1 (approx.)

The sugar was added to the mixture of peptone plus indicator, mixed thoroughly, then distributed in portions of 2 ml in sterile test tubes with
an inverted Durham tube, and then sterilized by autoclaving at 10/lb pressure for 10 minutes.

2. Reagents

2.1. Hydrogen peroxide:
Obtained from British Drug House (BDH) Company, England. It was prepared as 3% aqueous solution and used for catalase test.

2.3. Nitrate test reagents:
These were composed of two solutions.

Solution (A):
0.8% sulphanilic acid in acetic acid. This was prepared by mixing 0.4 ml of sulphanilic acid in 50 ml of 5N- acetic acid.

Solution (B):
0.5% dimethyl-α- nephthylamine in 5N-acetic acid.

2.4. Solutions:

2.4.1. Normal saline (0.85%):
It was prepared by dissolving 8.5 gm of sodium chloride in one liter distilled water then sterilized by autoclaving at 121°C for 15 min.
2.4.2. Phosphate buffered saline solution (PBSS)

It was prepared by dissolving the following ingredients:

- Sodium chloride 8 g
- Potassium chloride 0.2g
- Di Sodium hydrogen phosphate (Na2 Hpo4) 1.15g
- Potassium dihydrogen phosphate (KH2 PO4) 0.2g

The ingredients were dissolved in one liter of distilled water and sterilized at 121°C for 15 minutes.
2. Mass Cultivation of *C. pseudotuberculosis* using IBT Bioreactor:

Appendix Fig. 1: An illustration of Goettingen IBT bioreactor set-up for bacterial mass production (Böhnel, 1999).
3.2.3. Results of statistical analysis:

Appendix table 1: Means of abscesses in vaccinated and control animals

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Site of challenge</th>
<th>Prescap. L.N.</th>
<th>Lung</th>
<th>Liver</th>
<th>Mediastinal L. N.</th>
<th>Omentum</th>
<th>Precrural L.N.</th>
<th>Subcutaneous</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1(CW)</td>
<td>1.0000</td>
<td>0.1667</td>
<td>0.3333</td>
<td>2.6670</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>4.1670</td>
</tr>
<tr>
<td>G2(WC)</td>
<td>0.8000</td>
<td>0.2000</td>
<td>0.0000</td>
<td>1.6000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.2000</td>
<td>0.0000</td>
<td>3.5000</td>
</tr>
<tr>
<td>G3(T)</td>
<td>0.5000</td>
<td>0.0000</td>
<td>1.0000</td>
<td>8.5000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>10.0000</td>
</tr>
<tr>
<td>G4(CW+T)</td>
<td>1.0000</td>
<td>0.2500</td>
<td>0.0000</td>
<td>1.7500</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>3.0000</td>
</tr>
<tr>
<td>G5(WC+T)</td>
<td>0.8000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.8000</td>
</tr>
<tr>
<td>G6(control)</td>
<td>1.0000</td>
<td>0.2000</td>
<td>1.2000</td>
<td>3.6000</td>
<td>0.2000</td>
<td>0.1000</td>
<td>0.2000</td>
<td>0.4000</td>
<td>7.8000</td>
</tr>
</tbody>
</table>

L. N. = Lymph node

Means* followed by the same letter in each column are not significantly different (P > 0.05)

(Duncan Grouping)
3.3. Antimicrobial susceptibility results:

**Appendix table 2: Antibiotic sensitivity test results of the *C. pseudotuberculosis* isolates.**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Antibiotic conc.</th>
<th>Resistant &lt;12mm</th>
<th>Moderate 12—18 mm</th>
<th>Sensitive &gt;18mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10 µg</td>
<td>0/66 0.0%</td>
<td>14/66 21.2%</td>
<td>52/66 78.8%</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>5 µg</td>
<td>1/61 1.7%</td>
<td>16/61 26.2%</td>
<td>44/61 72.1%</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10 µg</td>
<td>0/65 0.0%</td>
<td>22/65 33.8%</td>
<td>43/65 66.2%</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>30 µg</td>
<td>1/36 2.8%</td>
<td>3/36 8.3%</td>
<td>32/36 88.9%</td>
</tr>
<tr>
<td>Rifampacin</td>
<td>2 µg</td>
<td>0/37 0.0%</td>
<td>8/37 21.6%</td>
<td>29/37 78.4%</td>
</tr>
<tr>
<td>Penicillin</td>
<td>1 iu</td>
<td>58/66 87.9%</td>
<td>8/66 12.1%</td>
<td>0/66 0.0%</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 µg</td>
<td>48/66 72.7%</td>
<td>18/66 27.3%</td>
<td>0/66 0.0%</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 µg</td>
<td>6/51 11.7%</td>
<td>34/51 66.7%</td>
<td>11/51 21.6%</td>
</tr>
<tr>
<td>Methicillin</td>
<td>1/37</td>
<td>2.7%</td>
<td>26/37 70.3%</td>
<td>10/37 27.0%</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>5 µg</td>
<td>38/50 76.0%</td>
<td>11/50 22.0%</td>
<td>1/50 2.0%</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>10 µg</td>
<td>3/66 4.5%</td>
<td>43/66 65.2%</td>
<td>20/66 30.3%</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>200 µg</td>
<td>0/15 0.0%</td>
<td>2/15 13.3%</td>
<td>13/15 86.7%</td>
</tr>
<tr>
<td>Cotrimexazole</td>
<td>25 µg</td>
<td>0/14 0.0%</td>
<td>3/14 21.4%</td>
<td>11/14 78.6%</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>30 µg</td>
<td>15/15 100%</td>
<td>0/15 0.0%</td>
<td>0/15 0.0%</td>
</tr>
<tr>
<td>Colistin</td>
<td>25 µg</td>
<td>15/15 100%</td>
<td>0/15 0.0%</td>
<td>0/15 0.0%</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>30 µg</td>
<td>31/33 93.9%</td>
<td>2/33 6.1%</td>
<td>0/33 0.0%</td>
</tr>
</tbody>
</table>
Chapter Three
Results

3.1. Results of field survey:

3.1.1. The prevalence of *C.pseudotuberculosis* among live and slaughtered sheep

Of the 54 samples of pyogenic aspirates collected from enlarged lymph nodes of live sheep at local animal markets in Nyala and 89 samples of abscess collected from sheep carcasses during meat inspection at Nyala slaughterhouse, *C. pseudotuberculosis* was isolated from 38 (70.4%) of the live sheep and 58 (65.2%) of the slaughtered sheep. The prevalence of CLA was 0.6% for live sheep and 1.7% for slaughtered sheep. The overall prevalence of disease in sheep was 1%. (Table 1).

3.1.2. The prevalence of *C.pseudotuberculosis* among live and slaughtered goats:

Of the 34 pyogenic aspirates collected from enlarged lymph nodes of live goats at local animal markets in Nyala and 6 samples of pus from infected lymph nodes collected from goats carcasses during meat inspection at Nyala slaughterhouse, *C. pseudotuberculosis* was isolated from 58 (65.2%) of live goats and 5 (83.3%) of slaughtered goats. The prevalence of CLA was 0.6% for live goats and 2.7% for slaughtered goats.
The overall prevalence of the disease in goats was 0.7% (Table 2).

3.1.3. Distribution of CLA lesions in sheep and goats:

Eighty nine lesions of CLA in inspected slaughtered sheep were distributed as follows: 58 (65.2%) in prescapular, 13 (14.6%) in precrural, 6 (6.7%) in parotid, 5 (5.6%) in supramammary, 1 (1.1%) in mandibular, 1 (1.1%) in mediastinal, 1 (1.1%) in mesenteric lymph nodes, 2 (2.2%) subcutaneous abscesses, 1 (1.1%) in liver and 1 (1.1%) in lung. Fifty two lesions of CLA in examined live sheep were distributed as follows: 15 (28.8%) in parotid, 13 (25%) in prescapular, 7 (13.5%) in mandibular, 3 (5.8%) in precrural lymph nodes and 14 (26.9%) subcutaneous abscesses. The overall distribution of 141 lesions in slaughtered and live sheep together were found to be 70 (50.4%), in prescapular 20 (14.9%) in parotid, 16 (11.3%) in precrural, 8 (5.7%) in mandibular, 5 (3.5%) in supramammary, 1 (0.7%) in mediastinal, 1 (0.7%) in mesenteric lymph nodes, 1 (0.7%) in liver, 1 (0.7%) in lung and 16 (11.3%) subcutaneous abscesses (Table 3).

Whereas in slaughtered goats, 6 lesions were found, 4 (66.7%) in parotid and 2 (33.3%) in prescapular lymph nodes. Thirty four lesions of CLA in live goats were distributed as follows: 17 (50%) in prescapular, 14 (41.2%) in parotid, 5 (14.7%) in mandibular, 4 (5.9%) in precrural lymph nodes and 4 (5.9%) subcutaneous abscesses. The overall distribution of 40 lesions
in slaughtered and life goats was 19 (47.5 %) in prescapular, 12 (30 %) in parotid, 4 (10 %) in precrural, 5 (12.5 %) in mandibular lymph nodes, and 4 (10 %) subcutaneous abscesses (Table 4).

3.1.4. Identification of bacteria:

The criteria used for the identification of isolates were as follows:

3.1.4.1. Morphology and Staining:

The organism appeared in stained smears as Gram-positive, small rods, tending to form clumps resembling Chinese letters in arrangement. It was non-motile, non-spore-forming.

3.1.4.2. Cultural characteristic:

3.1.4.2.1. On blood agar medium:

After 48 hours at 37°C, growth was in the form of small tiny colonies (about 1mm in diameter). Colonies were white, opaque, flat and waxy in consistency. They splattered on flaming and were easily pushed across the agar surface.

3.1.4.2.2. Brain heart infusion broth:

The growth extended on the sides of the tube, forming a pellicle and granular sediment with no general turbidity.
Table 1: Number of live and slaughtered sheep with abscesses and CLA.

<table>
<thead>
<tr>
<th></th>
<th>Total No. of animals examined</th>
<th>No. of animals with abscesses</th>
<th>% of animals with abscesses</th>
<th>No. of +ve for C. pseudotuberculosis</th>
<th>% of +ve for C. pseudotuberculosis among affected animals</th>
<th>% of +ve for C. pseudotuberculosis among total animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live Sheep</td>
<td>6560</td>
<td>52</td>
<td>0.8 %</td>
<td>38</td>
<td>73.1 %</td>
<td>0.6 %</td>
</tr>
<tr>
<td>Slaughtered Sheep</td>
<td>3324</td>
<td>89</td>
<td>2.8 %</td>
<td>58</td>
<td>65.2 %</td>
<td>1.7 %</td>
</tr>
<tr>
<td>Total</td>
<td>9884</td>
<td>141</td>
<td>1.4 %</td>
<td>96</td>
<td>68.1 %</td>
<td>1 %</td>
</tr>
</tbody>
</table>

CLA: Caseous lymphadenitis
### Table 2: Numbers of live and slaughtered goats with abscesses and CLA.

<table>
<thead>
<tr>
<th></th>
<th>Total No. of animals examined</th>
<th>No. of animals with abscesses</th>
<th>% of animals with abscesses</th>
<th>No. of +ve C. pseudotuberculosis</th>
<th>% of +ve for C. pseudotuberculosis among affected animals</th>
<th>% of +ve for C. pseudotuberculosis among total animal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Live Goats</strong></td>
<td>4450</td>
<td>34</td>
<td>0.8%</td>
<td>27</td>
<td>76.5%</td>
<td>0.6%</td>
</tr>
<tr>
<td><strong>Slaughtered Goats</strong></td>
<td>184</td>
<td>6</td>
<td>3.3%</td>
<td>5</td>
<td>83.3%</td>
<td>2.7%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>4634</td>
<td>40</td>
<td>0.86%</td>
<td>32</td>
<td>80%</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

**CLA:** Caseous lymphadenitis
Table 3: Distribution of CLA lesions in live and slaughtered sheep.

<table>
<thead>
<tr>
<th></th>
<th>Total No. of lesions</th>
<th>Prescapular L. N.</th>
<th>Parotid L. N.</th>
<th>Precrural L. N.</th>
<th>Mandibular L. N.</th>
<th>Mediastinal L. N.</th>
<th>Mesenteric L. N.</th>
<th>Supramammary L. N.</th>
<th>liver</th>
<th>lung</th>
<th>Subcutaneous abscess</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slaughtered sheep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>89</td>
<td>58</td>
<td>6</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>65.2%</td>
<td>6.7%</td>
<td>14.6%</td>
<td>1.1%</td>
<td>1.1%</td>
<td>1.1%</td>
<td>5.6%</td>
<td>1.1%</td>
<td>1.1%</td>
<td>2.2</td>
</tr>
<tr>
<td>Live sheep</td>
<td>52</td>
<td>13</td>
<td>15</td>
<td>3</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>25%</td>
<td>28.8%</td>
<td>5.8%</td>
<td>13.5%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>26.9%</td>
</tr>
<tr>
<td>Total</td>
<td>141</td>
<td>71</td>
<td>21</td>
<td>16</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Over all %</td>
<td>%</td>
<td>50.4%</td>
<td>14.9%</td>
<td>11.3%</td>
<td>5.7%</td>
<td>0.7%</td>
<td>0.7%</td>
<td>3.5%</td>
<td>0.7%</td>
<td>0.7%</td>
<td>11.3%</td>
</tr>
</tbody>
</table>

L.N : Lymph node
CLA: Caseous lymphadenitis
Table 4: Distribution of CLA lesions in live and slaughtered goats.

<table>
<thead>
<tr>
<th></th>
<th>Total No. of lesions</th>
<th>Prescapular L. N.</th>
<th>Parotid L. N.</th>
<th>Precrural L. N.</th>
<th>Mandibular L. N.</th>
<th>Subcutaneous abscess</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live goats</td>
<td>38</td>
<td>17</td>
<td>8</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>-</td>
<td>44.7%</td>
<td>21.1%</td>
<td>10.5%</td>
<td>13.2%</td>
</tr>
<tr>
<td>Slaughtered goats</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>-</td>
<td>33.3 %</td>
<td>66.7 %</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>19</td>
<td>12</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Over all %</td>
<td>-</td>
<td>-</td>
<td>43.2 %</td>
<td>27.3%</td>
<td>9.1%</td>
<td>11.4%</td>
</tr>
</tbody>
</table>

L.N : Lymph node  
CLA: Caseous lymphadenitis
3.1.4.3. Biochemical tests:

Isolates were positive to produce catalase, hydrolysed urea, most of it were negative to reduce nitrate to nitrite. The isolates fermented glucose, fructose, mannose, maltose, galactose and were negative for salcin, trehalose and starch.

3.2. Results of vaccination trials:

3.2.1. Serological responses to vaccines:

The groups of animals were monitored for antibody titres by agglutination tests. The animals vaccinated with cell wall, whole cell, cell wall+ toxoid and whole cell+ toxoid showed serological responses. Titres began to increase and reached the primary peaks two weeks post vaccination, then declined. Other secondary peaks of the titres were observed one week after the booster doses. It was observed that in the four groups of animals vaccinated with cell wall, whole cell, cell wall + toxoid and whole cell+ toxoid the results of the agglutination tests showed similar pattern either in duration or degree of response, which could be considered of equal magnitude (i.e.) a rise in agglutination titres was evident in the following first week, and by the end of the second week it began to fall and reached its lowest level by the end of the fourth week (Figure 5). The titre in sheep vaccinated with toxoid and non-vaccinated control animals were unchanged until
challenge when inoculated s/c using virulent \textit{C. pseudotuberculosis} CP41N, then the animals began to respond and the titres reached their peaks 2 weeks after s/c challenge

3.2.2. Results of challenge:

After three days of subcutaneous challenge with \((5.1 \times 10^8 \text{ CFU/ml})\) of \textit{C. pseudotuberculosis} strain CP41N of all sheep groups, three animals from the control group died. There were no abscesses at the sites of challenge or internal organs, but there were acute inflammation and distinct enlargements of the prescapular lymph nodes draining the site of challenge (Figure 6). Six vaccinated animals died from pneumonia before challenge, but there were no deaths among vaccinated animals after challenge.

Four weeks after challenge all remaining controls and vaccinated sheep were slaughtered. The distribution of abscesses and lesions in different organs (Figures 6, 7 and 8) were presented in (Table 5). Pus from the lesions were Positive for \textit{C. pseudotuberculosis} upon culture.

The protection of the different vaccines were found to be 32.3\%, 37.5\%, 37.5\%, 45.3\% and 75\% in animals vaccinated with cell wall, whole cell, toxoid, cell wall + toxoid and whole cell + toxoid respectively (Table 6).
Fig. 4: Agglutination titres of sheep injected with the different *C. pseudotuberculosis* vaccines.
Fig. 5: Abscess at site of injection in a control sheep challenged with *C. pseudotuberculosis*
Fig. 6: Presacpular lymph nodes of a control sheep that had died after challenge with *C. pseudotuberculosis*. The lymph node on the right is the one that draining challenge injection site, the lymph node on the left is the one draining the other side.
Table 5: Abscesses in control and vaccinated sheep after challenge with *C. pseudotuberculosis*.

<table>
<thead>
<tr>
<th>Group of the Animal</th>
<th>Site of challenge</th>
<th>Prescapular L. N.</th>
<th>Lung</th>
<th>Liver</th>
<th>Mediastinal L. N.</th>
<th>Omentum</th>
<th>Precrural L. N.</th>
<th>Subcut absces.</th>
<th>Total</th>
<th>% of infected organs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1, Cell Wall (CW) vaccine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>37.5%</td>
</tr>
<tr>
<td>Animal 2</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>2</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 5</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 6</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 7*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25</td>
<td>27.1%</td>
</tr>
<tr>
<td><strong>Group 2, Whole Cell (WC) vaccine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 1</td>
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<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>37.5%</td>
</tr>
<tr>
<td>Animal 2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>-</td>
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</tr>
<tr>
<td>Animal 3</td>
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<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 5*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>12.5%</td>
</tr>
<tr>
<td>Animal 7*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14</td>
<td>25%</td>
</tr>
</tbody>
</table>

* Animals died before challenge

** Animals died within 3 days after challenge
Table. 5 (Cont.): Abscesses in control and vaccinated sheep after challenge with *C. pseudotuberculosis*.

<table>
<thead>
<tr>
<th>Groups of the animal</th>
<th>Site of Challenge</th>
<th>Prescapular L. N</th>
<th>Lung</th>
<th>Liver</th>
<th>Mediastinal L. N</th>
<th>Omentum</th>
<th>Precrural L. N</th>
<th>Subcut absces</th>
<th>Total</th>
<th>% of infected organs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 3, Toxoid (T) vaccine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 1*</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 2*</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 3</td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 4*</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 5*</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 6</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>6</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 7*</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>25%</td>
</tr>
</tbody>
</table>

| **Group 4, Cell Wall+Toxoid (CW+T) vaccine** |                   |                 |      |       |                 |         |                |                |       |                     |
| Animal 1             |                   | 1               | 0    | 0     | 0               | 0       | 0              | 0              | 0     | 1                   | 12.5% |
| Animal 2*            |                   | -               | -    | -     |                 |         |                |                |       |                     |
| Animal 3             |                   | 1               | 0    | 0     | 6               | 0       | 0              | 0              | 7     | 25%                 |
| Animal 4*            |                   | -               | -    | -     |                 |         |                |                |       |                     |
| Animal 5             |                   | 1               | 0    | 0     | 0               | 0       | 0              | 0              | 1     | 12.5%               |
| Animal 6             |                   | 1               | 1    | 0     | 1               | 0       | 0              | 0              | 3     | 37.5%               |
| Animal 7*            |                   | -               | -    | -     |                 |         |                |                |       |                     |
| **Total**            |                   | 4               | 1    | 0     | 7               | 0       | 0              | 0              | 12    | 21.9%               |

* Animals died before challenge  
** Animals died within 3 days after challenge
<table>
<thead>
<tr>
<th>Groups of the animal</th>
<th>Site of challenge</th>
<th>Prescapular L. N</th>
<th>Lung</th>
<th>Liver</th>
<th>Mediastinal L. N.</th>
<th>Omentum</th>
<th>Precrural L. N</th>
<th>Subcut. absces.</th>
<th>Total</th>
<th>% of infected organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 5, Whole Cell+Toxoid (WC+T) vaccine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>12.5%</td>
</tr>
<tr>
<td>Animal 2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>12.5%</td>
</tr>
<tr>
<td>Animal 3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>12.5%</td>
</tr>
<tr>
<td>Animal 4*</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Animal 5*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Animal 6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Animal 7</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>1</td>
<td>12.5%</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>10%</td>
</tr>
<tr>
<td>Group 6, Control.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 1**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Animal 2**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Animal 3**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Animal 4</td>
<td>1</td>
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<td>1</td>
<td>13</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>37.5%</td>
</tr>
<tr>
<td>Animal 5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>25%</td>
</tr>
<tr>
<td>Animal 6</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>62.5%</td>
</tr>
<tr>
<td>Animal 7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>37.5%</td>
</tr>
<tr>
<td>Animal 8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>37.5%</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>55</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>37</td>
<td>40%</td>
</tr>
</tbody>
</table>

* Animals died before challenge  
** Animals died within 3 days after challenge
Table 6: Protection of vaccinated animals.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Total No. of organs</th>
<th>Total No. of infected organs</th>
<th>Percentage of infected organs</th>
<th>Percentage of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1, Cell Wall (CW) vaccine</td>
<td>48</td>
<td>13</td>
<td>27.1%</td>
<td>32.3%</td>
</tr>
<tr>
<td>Group 2, Whole Cell(WC) vaccine</td>
<td>32</td>
<td>8</td>
<td>25%</td>
<td>37.5%</td>
</tr>
<tr>
<td>Group 3, Toxoid (T) vaccine</td>
<td>16</td>
<td>4</td>
<td>25%</td>
<td>37.5%</td>
</tr>
<tr>
<td>Group 4, Cell Wall+Toxoid (CW+T)</td>
<td>32</td>
<td>7</td>
<td>21.9%</td>
<td>45.3%</td>
</tr>
<tr>
<td>Group 5, Whole Cell+Toxoid (WC+T)</td>
<td>40</td>
<td>5</td>
<td>10%</td>
<td>75%</td>
</tr>
<tr>
<td>Group 6, Control.</td>
<td>40</td>
<td>16</td>
<td>40%</td>
<td>0%</td>
</tr>
</tbody>
</table>
3.2.3. Results of statistical analysis:

3.2.3.1. Description:

Mean values of abscesses of vaccinated and control animals were presented in table 7. The distribution of abscesses in vaccinated and control animals were shown in figures 7 and 8.

Table 7: Mean values of abscesses in vaccinated and control animals

<table>
<thead>
<tr>
<th>Total number of abscess detected</th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>95% Confidence Interval for Mean</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 (CW)</td>
<td>6</td>
<td>4.167</td>
<td>3.25064</td>
<td>1.32707</td>
<td>.7553 – 7.5780</td>
<td>2.00</td>
<td>10.00</td>
</tr>
<tr>
<td>G2 (WC)</td>
<td>5</td>
<td>2.600</td>
<td>2.30217</td>
<td>1.02956</td>
<td>-.2585 – 5.4585</td>
<td>.00</td>
<td>6.00</td>
</tr>
<tr>
<td>G3 (T)</td>
<td>2</td>
<td>10.000</td>
<td>5.65685</td>
<td>4.00000</td>
<td>-40.8248 – 60.8248</td>
<td>6.00</td>
<td>14.00</td>
</tr>
<tr>
<td>G4 (CW+T)</td>
<td>4</td>
<td>3.000</td>
<td>2.82843</td>
<td>1.41421</td>
<td>-1.5007 – 7.5007</td>
<td>1.00</td>
<td>7.00</td>
</tr>
<tr>
<td>G5 (WC+T)</td>
<td>5</td>
<td>.8000</td>
<td>.44721</td>
<td>.20000</td>
<td>.2447 – 1.3553</td>
<td>.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>14.400</td>
<td>21.13764</td>
<td>9.45304</td>
<td>-11.8459 – 40.6459</td>
<td>2.00</td>
<td>52.00</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>5.4074</td>
<td>9.88884</td>
<td>1.90311</td>
<td>1.4955 – 9.3193</td>
<td>.00</td>
<td>52.00</td>
</tr>
</tbody>
</table>
Fig. 7: Histograms showing number of abscesses in control and vaccinated animals after challenge with *C. pseudotuberculosis*.

Fig. 8: A histogram showing distribution of abscesses in control and vaccinated animals after challenge with *C. pseudotuberculosis*.
3.2.3.2. Analysis of variance (ANOVA):

The data was found to be not normally distributed, so it was transformed using log transformation and analysis of variance (ANOVA) was used to evaluate and compare the findings of the vaccinated and control animals. The results showed that the mean difference between groups was significant (P-value < 0.017) (table 7).

3.2.3.3. Least significant difference (LSD):

When the least significant difference (LSD) was applied, it showed that the mean difference was significant (P-value < 0.05) when group 5 was compared with group 6 and group 3 and less significant when compared with group 1, 2 and 4, probably due to the small sample sizes (animals that died needed to be excluded).

Group 5 looked best and group 3 seemed to be the same as the control group, the others seemed to have intermediate effectiveness (Table 8).

From the analysis it could be concluded that group 5 vaccinated with (WC +T) was the most protected group.

<table>
<thead>
<tr>
<th>Table 8: Analysis of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of abscess detected</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>Between Groups</td>
</tr>
<tr>
<td>Within Groups</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
Table 9: Multiple comparisons between abscesses of vaccinated and control animals.

Dependent Variable: Total number of abscess detected

LSD

<table>
<thead>
<tr>
<th>(I) Treatment groups</th>
<th>(J) Treatment groups</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 (CW)</td>
<td>G 2 (WC)</td>
<td>1.5667</td>
<td>1.66291</td>
<td>.357</td>
<td>-1.9021 5.0354</td>
</tr>
<tr>
<td>G 3 (T)</td>
<td>G 4 (CW+T)</td>
<td>-5.8333*</td>
<td>2.24227</td>
<td>.017</td>
<td>-10.5106 -1.1560</td>
</tr>
<tr>
<td>G 4 (OW+T)</td>
<td>G 5 (WC+T)</td>
<td>3.3667</td>
<td>1.66291</td>
<td>.056</td>
<td>-.1021 6.8354</td>
</tr>
<tr>
<td>G 5 (WC+T)</td>
<td>Control</td>
<td>-.8333</td>
<td>1.77267</td>
<td>.643</td>
<td>-4.5311 2.8644</td>
</tr>
<tr>
<td>G 2 (WC)</td>
<td>G1 (CW)</td>
<td>-1.5667</td>
<td>1.66291</td>
<td>.357</td>
<td>-5.0354 1.9021</td>
</tr>
<tr>
<td>G 3 (T)</td>
<td>G 4 (OW+T)</td>
<td>-7.4000*</td>
<td>1.84221</td>
<td>.830</td>
<td>-4.2428 3.4428</td>
</tr>
<tr>
<td>G 4 (OW+T)</td>
<td>G 5 (WC+T)</td>
<td>1.8000</td>
<td>1.73686</td>
<td>.312</td>
<td>-1.8230 5.4230</td>
</tr>
<tr>
<td>G 5 (WC+T)</td>
<td>Control</td>
<td>-2.4000</td>
<td>1.84221</td>
<td>.207</td>
<td>-6.2428 1.4428</td>
</tr>
<tr>
<td>G 3 (T)</td>
<td>G1 (OW)</td>
<td>5.8333*</td>
<td>2.24227</td>
<td>.017</td>
<td>1.1560 10.5106</td>
</tr>
<tr>
<td>G 2 (WC)</td>
<td>G 4 (OW+T)</td>
<td>7.4000*</td>
<td>1.84221</td>
<td>.830</td>
<td>2.6072 12.1928</td>
</tr>
<tr>
<td>G 5 (WC+T)</td>
<td>Control</td>
<td>5.0000*</td>
<td>2.37829</td>
<td>.039</td>
<td>9.9610 11.9610</td>
</tr>
<tr>
<td>G 4 (OW+T)</td>
<td>G1 (CW)</td>
<td>-1.1667</td>
<td>1.77267</td>
<td>.518</td>
<td>-4.8644 2.5311</td>
</tr>
<tr>
<td>G 2 (WC)</td>
<td>G 3 (T)</td>
<td>7.0000*</td>
<td>1.84221</td>
<td>.008</td>
<td>2.0390 11.9610</td>
</tr>
<tr>
<td>G 3 (T)</td>
<td>G 5 (WC+T)</td>
<td>2.2000</td>
<td>1.84221</td>
<td>.246</td>
<td>-1.6428 6.0428</td>
</tr>
<tr>
<td>G 5 (WC+T)</td>
<td>Control</td>
<td>-2.0000</td>
<td>1.94186</td>
<td>.315</td>
<td>-6.0507 2.0507</td>
</tr>
<tr>
<td>G 5 (WC+T)</td>
<td>G1 (CW)</td>
<td>-3.3667</td>
<td>1.66291</td>
<td>.056</td>
<td>-6.8354 .1021</td>
</tr>
<tr>
<td>G 2 (WC)</td>
<td>G 3 (T)</td>
<td>-1.8000</td>
<td>1.73686</td>
<td>.312</td>
<td>-5.4230 1.8230</td>
</tr>
<tr>
<td>Control</td>
<td>G1 (OW)</td>
<td>.8333</td>
<td>1.77267</td>
<td>.643</td>
<td>-2.8644 4.5311</td>
</tr>
<tr>
<td>G 2 (WC)</td>
<td>G 3 (T)</td>
<td>2.4000</td>
<td>1.84221</td>
<td>.207</td>
<td>-1.4428 6.2428</td>
</tr>
<tr>
<td>G 3 (T)</td>
<td>G 4 (OW+T)</td>
<td>-5.0000*</td>
<td>2.37829</td>
<td>.048</td>
<td>-9.9610 -.0390</td>
</tr>
<tr>
<td>G 4 (OW+T)</td>
<td>G 5 (WC+T)</td>
<td>2.0000</td>
<td>1.94186</td>
<td>.315</td>
<td>-2.0507 6.0507</td>
</tr>
</tbody>
</table>

*: The mean difference is significant at the .05 level.
3.3. Antimicrobial susceptibility results:

It was found that 86.7% of the isolates of *C.pseudotuberculosis* tested were highly sensitive to nitrifurantoin, 78.8% to chloramphenicol, 78.8% to rifampacin, 78.6% to cotrimoxazole, 72.1% to erythromycin and 66.2% to ampicillin.

On the other hand 70.3% of the isolates were moderately sensitive to methicillin, 66.7% to kanamycin, 65.2 to gentamycin and 59.1% to tetracycline. Whereas 100% of the isolates were resistant to nalidixic acid and colistin, 93.3% to novobiocin, 87.9% to penicillin, 76% to cloxacillin and 72.7% to Streptomycin (Figure 9).

3.4. Results of PCR product:

By passing the products of PCR in gel, strains of *C. pseudotuberculosis* showed the same designated molecular size (816 bp) for the primer targeting the 16S rRNA gene of *C. pseudotuberculosis*, yet negative control (reaction without template DNA) showed no results. Figures 10, 11, 12 and 13 show results of samples obtained from different strains.
Fig. 4: Susceptibility of C. pseudotuberculosis to selected antibiotics

<table>
<thead>
<tr>
<th>Type of antibiotics</th>
<th>Percentage of C. pseudotuberculosis strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resist</td>
<td>Moderate</td>
</tr>
<tr>
<td>Sensitive</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 9: Antibiotic susceptibility tests results of the C. pseudotuberculosis isolates
Fig. 10: Results of PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis for strains: CP199N (2), CP201N (3), CP152N (4), CP41N (5), negative control (6), strains: CP275N (7) and CP216N (8), whereas DNA marker ladder is in lane (1)

Fig. 11: Results of PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis for strains: CP165N (2), CP230N (3), CP209N (4), CP229N (5), CP187N (6), CP180N (7), CP160N (8), CP221N (9), CP148N (10), CP162N (11), CP196N (12), CP322N (13), CP144N (14), CP313N (15), CP153N (16), whereas DNA marker ladder is in lane (1)
Fig. 12: Results of PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis for strains CP41N (2), CP144N (3), CP148N (4), CP153N (5), CP160N (6), CP162N (7), CP196N (8), CP214N (9), CP221N (10), CP313N (11), CP322N (12) and negative control (13), whereas DNA marker ladder is in lane (1).

Fig. 13: Results of PCR amplification of *C. pseudotuberculosis* 16S rDNA gene sequences on agarose gel-electrophoresis for strains CP214N (2), negative control (3), CP41N (4), CP64N (5), CP65N (6), CP229N (7), CP203N (8), CP47aW (9), CP47bW (10), CP187N (11) and CP180N (12) whereas DNA marker ladder is in lane (1).
Chapter Four

Discussion

Caseous lymphadenitis causes great economical losses in sheep and goats’ production due to condemnation of animals or parts of carcasses because of the infection. It affects animal trade as detection of the external disease in few animals leads to rejection of whole sheep export shipment and internal lesions may not be detected till after slaughter. Surgical treatment of the disease is not successful as it causes extensive fibrosis at the site of infection and the disease may recurs. Moreover, lancing and draining the superficial abscesses contaminates the skin and the surrounding environment. The unguided use of antibiotics increases the problem of antibiotic resistance. Thus, to combat the disease efforts should be directed to produce a protective vaccine against the disease.

Several vaccines were developed in order to find a reliable one for control the disease in sheep and goats. Different antigen were used but those vaccines showed varied degrees of protection.

The aim of this study was to determine prevalence of the disease among sheep and goats in South Darfur State and to produce mass culture of
C. pseudotuberculosis with IBT bioreactor to be used for vaccine production.

In this study physical examination by palpation of external lymph nodes of 6560 live sheep and 4450 live goats revealed 34 (0.82%) and 54 (0.76%) had enlarged lymph nodes in both of them respectively. In slaughtered sheep and goats abscesses and pyogenic lesions due to C. pseudotuberculosis were found in 58 (1.7%) of 3324 carcasses of sheep and in 5(2.7%) of 184 carcasses of goats. Khalid(1971) reported 7.4% prevalence of CLA in slaughtered sheep at Omdurman slaughterhouse, whereas Elgaddal (1997) reported a prevalence of 1.2% among live sheep and 3.2 % in slaughtered sheep due to C. pseudotuberculosis in the same area of our study. Higher prevalence rates of 6.4% and 7.1% in sheep and goats respectively were reported in the same area by Musa (1998). The lower prevalence rates observed in this study might be due to the increased awareness of animal traders about the disease and the selection of apparently disease free animals for he local markets and for export. They were informed that animal collected should not show abscesses.

Pure C. pseudotuberculosis was isolated from 58 (70.4%) of 89 lesions from sheep during meat inspection in Nyala slaughterhouse, this rate is higher than that stated by El gaddal (1997) who had isolated pure C.
*pseudotuberculosis* from 51.8% sheep pus specimens at Nyala slaughterhouse, and also higher than that reported by Khalid (1971) who isolated *C. pseudotuberculosis* from 54.6% of 183 sheep abscesses collected from Omdurman slaughterhouse. Hence, our findings are supporting the idea that *C. pseudotuberculosis* is the predominant bacteria involved in sheep lymphadenitis and abscesses in the Sudan. Yet, in Khartoum State many investigators (Hamad *et al.*, 1992; Noura Karmalla, 1997; Sara Bihary, 2002 and Nasreen Musa, 2009) isolated *Staphylococcus spp.* from 73.9%, 96.5%, 48% and 68.8% of sheep respectively. They attributed the increased prevalence rate of *Staphylococcus aureus subsp. anaerobius* in sheep abscesses in Khartoum State to the practice of sheep fattening prior to slaughter or export. It is clear that *C. pseudotuberculosis* is predominant in the production areas where seep and goats are grazed in common fields, whereas *S. aureus subsp. anaerobius* is more frequently where animals are subjected to supplemented feeding for fattening in Khartoum State before slaughter. This in turn may lead to the incrimination of substances in rations that are used for fattening as a predisposing factors. Most sheep herders in Darfur and Kordofan believe that addition of salt to rations predisposes sheep to abscess formation. It is known that Staphylococci could tolerate up to 15% of salt concentration. Never the less,
this observation needs to be experimentally verified to put it in a scientific context, and then rations for sheep fattening could be modified for prevention measures.

In this study it was found that the most frequently infected lymph node in sheep was the prescapular lymph node and this is in agreement with many findings reported previously (Woodruff and Oxer, 1929; Gameel, 1974; Khalid, 1971; Ayers, 1977; Williamson and Narin, 1980; Kuria and Nagatia, 1990; Gilmour, 1990; Musa, 1998). Elgaddal (1997) attributed the frequent occurrence of abscesses and pyogenic lesions in the prescapular lymph node to wounds caused by thorns and sharp objects during grazing in wooded Savannah pastures. A total rate of 8.2% of pus samples from carcasses or aspirated from live sheep were negative when bacteriologically cultured. This is lower than the 15% rate reported by Elgaddal (1997) but higher than the 5% and 2.9% rates reported by Khalid (1971) and Hamad et al. (1992) respectively.

Confluent growth culture of C. pseudotuberculosis culture was successfully obtained using IBT bioreactor for the first time in Sudan, which showed the possibility of mass production of good quality culture and in short time. Our results were in agreement with those of Sulieman (2001) using the same parameters (pH 8, temperature 31°C and dilution rate 0.02 h⁻¹)
for optimum growth of *C. pseudotuberculosis* using IBT bioreactor with some modifications.

Five types of vaccines were tried to immunize sheep against CLA. The results showed that whole cell + toxoid (WC+T) was the most effective one among these vaccines. A significant reduction (P < 0.05) in abscess formation was noticed after challenge with the *C.pseudotuberculosis* in sheep vaccinated with this vaccine compared with unvaccinated animals controls. The protection percentage for the group vaccinated with this vaccine reached 75% compared with 32.3%, 37.5%, 37.5% and 45.3% for groups vaccinated with cell wall, whole cell, toxoid and cell wall + toxoid respectively. These results were in agreement with the findings of Simon-Valencia (1992) who reported that defatted whole cells of *C.pseudotuberculosis* with toxoid gave best but not complete protection to challenged mice, and with those of Burrell (1978b) who recommended the inclusion of bacterial cells in vaccines for control of CLA. However, Eggleton *et al.* (1991a) postulated that the addition of whole cells did not improve the protective potency of *C. pseudotuberculosis* toxoid vaccines, yet they illustrated that as the vaccine which was used in their trials, had been prepared from crude toxoid, and could therefore contain soluble antigens other than exotoxin, their results did not preclude the possibility that more
than one antigen was responsible for the protection that had been conferred.

In a previous study it was found that cell wall + toxoid vaccine produced a protection percentage of 65.2% in goats (Abdel Wahab 2000), this rate is higher than the one observed in the present study in sheep(45.3%) using the same type of vaccine possibly due to species variation in immunological response.

In this study sheep vaccinated with toxoid alone (T) were susceptible to infection similar to the control animals. Five of seven animals in this group died before challenge, i.e. only two animals remained which makes statistical evaluation of vaccination in this group unreliable.

In the present study the challenge dose of 5.1X10^8 CFU/ml of *C. pseudotuberculosis* inoculated subcutaneously was higher than the natural infection with *C. pseudotuberculosis* through the skin. *C. pseudotuberculosis* may be transmitted to sheep by contact of skin abrasions with contaminated soil or fomites (Hein and Cargill, 1981). A gram of pus may contain 10^6 to 10^7 CFU/ml of *C. pseudotuberculosis* (Augustine *et al.*, 1982), however, as this pus becomes dried and dispersed in soil or on fomites, the concentration would decrease, therefore, a natural challenge dose would be less than 5.1X10^8 CFU/ml of *C. pseudotuberculosis*.

Death of control animals after challenge due to acute toxicity was
reported by Nairn et al. (1977). The results of this study confirm this observation as 3 animals in the control group died within 24 hours after challenge.

The development of suppurative lesions at the site of challenge in control and vaccinated animals with varied degrees was reported by Nairn et al. (1977) and our findings are in agreement with this.

Comparison of five serological tests, including agglutination test, for diagnosis of the disease, showed that no test was 100% specific and sensitive (Shigidi, 1979). In this study we used the bacterial agglutination test for monitoring the humeral response in vaccinated then challenged animals, and the results obtained were similar to those of Shigidi (1979).

Results of susceptibility of *C. pseudotuberculosis* to antimicrobial agents were in agreement with that of Lipsky et al. (1982) and Muckie and Gyles (1982, 1983) except that our isolates were resistant to penicillin. We agreed with Judson and Songer (1991) but disagreed with them in sensitivity to penicillins and resistance to nitrofurantoin. Our findings in agreement with those of El gaddal (1997) and that reported by Musa (1998) but differ in that he found it sensitive to penicillins.

Resistance to penicillins is a growing problem which showed
in this study to reach 87.9% this findings is similar to that reported by Grag et al., (1985). Location of the CLA organism inside macrophages suggests that lipophilic drugs such as the macrolides would be more effective than penicillins, and that prolonged administration would be required for effective treatment (Prescott and Baggot, 1988).

PCR targeting the 16S rRNA gene showed positive results for all isolates previously known as *C. pseudotuberculosis*, and this is in agreement with Çetinkaya et al. (2002), putting in account that it could give the same results with *C. ulcerans*. This degree of similarity of *C. pseudotuberculosis* with *C. ulcerans* was also noted by Barksdale et al. (1981) who reported that phospholipase D activity has been found in strains exhibiting the biochemical properties characteristic of *C. pseudotuberculosis* or of *C. ulcerans* and in no other species of *Corynebacterium*. Although *C. ulcerans* is known to cause diphtheria-like disease in humans and is also important in cattle, the presence of this species in small ruminants has not been noted in the literature. Some biochemical tests (trehalose, maltotriose), have been reported to distinguish *C. pseudotuberculosis* from *C. ulcerans* (Hommez et al., 1999). A multiplex PCR (mPCR) assay targeting three genes of this bacterium: the 16S rRNA gene, rpoB and pld was developed by Pacheco et al. (2007) They reported that their mPCR assay was specific
enough to differentiate *C. pseudotuberculosis* from *C. ulcerans*. It was not possible to use this method in this study due to difficulties in obtaining the primers.
Chapter Five

Conclusions and Recommendations

Conclusions:

From this study we came to the following conclusions:

- Caseous lymphadenitis is prevalent in South Darfur State, West Sudan.

- Accurate diagnosis for disease by palpation in live animals is not possible, there is no 100% specific and sensitive serological test. In this study PCR was used for characterization of *C.pseudotuberculosis* isolates, but the use of the new multiplex PCR for diagnosis of the disease by using blood will be more useful for its diagnosis.

- The whole cell and toxoid vaccine produced with the IBT bioreactor resulted in high degree of protection.

- The causative organism (*C. pseudotuberculosis*) is sensitive to nitrifurantoin, chloramphenicol, rifampacin, cotrimoxazole, erythromycin, ampicillin.methicillin, kanamycin, gentamycin tetracycline but resistant to nalidixic acid, colistin, novobiocin, penicillin, cloxacillin and streptomycin. It is clear that resistance to
penicillins emerged lately.

**Recommendations:**

- Trials for using the whole cell +toxoid vaccine in the field under natural conditions for determination its efficacy in protection against CLA is recommended.

- As it is evident that both of CLA and Morel’s diseases exist in Sudan and should be dealt with simultaneously. A combined vaccine against them composed of this vaccine and other appropriate one against Morel’s disease could be tried.

- Careful use of antibiotics, infection control measures and periodic surveillance for the antibiotic resistance studies could provide a useful means for controlling this disease.
References


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